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BioMaH

a cura di

Julietta V. Rau e Antonio Ravaglioli



PROCEEDINGS

1ST BIENNIAL CONFERENCE



BIOMATERIALS FOR HEALTHCARE

BIOMATERIALS FOR TISSUE AND GENETIC ENGINEERING AND
THE ROLE OF NANOTECHNOLOGY

a cura di
Julietta V. Rau e Antonio Ravaglioli

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PROF. M. ALINI (SWITZERLAND)

AO Research Institute, Davos

Mauro Alini graduated in Chemistry from the University of Lausanne (Switzerland) in 1983. Since then he has been involved in connective tissue research, starting from his Ph.D. research work, done at the Laboratory of Cellular Pathology in Locarno (Switzerland), which focused on the isolation and characterization of proteoglycans extracted from both normal human mammary gland and carcinomas thereof. In September 1988, he joined the Joint Diseases Laboratory (under Dr. A. R. Poole's direction) at the Shriners Hospital in Montreal to work on quantitative and qualitative changes in extracellular matrix proteins (particularly proteoglycans and collagens) of the growth plate tissue before and at the time of cartilage matrix calcification during endochondral bone formation. In January 1995, he was appointed as an Assistant Professor at the Division of Orthopaedic Surgery of the McGill University (Chair Prof. M. Aebi) and head of the Biochemistry Unit of the Orthopaedic Research Laboratory, working to develop new biological ap-



proaches to treating intervertebral disc damage. Since July 2000, he is in charge of the Musculoskeletal Regeneration Program at the AO Research Institute (Davos, Switzerland), focusing on cartilage, bone and intervertebral disc tissue engineering. Since September 2009 is also the Vice-Director of the same Research Institute. He received the Marshall R. Urist Award in 2015 from the Orthopaedic Research Society (USA).

CELLS HOMING: HYDROGELS FOR CHEMOKINES DELIVERY

Cell therapy approaches, combining injectable materials and cells or growth factors/chemokines, represent a minimally invasive treatment for degenerated intervertebral disc (IVD). Recently, we have developed a hydrogel platform based on hyaluronan backbone. These include thermoreversible hydrogel and injectable tyramine modified

hyaluronic acid (HA-Tyr). Thermoreversible hydrogel provides easy injectability (low viscosity at room temperature) together with a mild gelling mechanism (physical cross-linking above 32°C), while tyramine modified hyaluronan viscoelastic properties, in vitro swelling and enzymatic digestion profiles of the crosslinked hydrogels can be precisely tuned via the degree of substituted Tyr on hyaluronan.

Recent studies have shown that human MSCs have the capability to survive within the disc. Indeed, pilot human studies have also been started. It is, however, not yet clear whether MSCs directly or indirectly contribute to the healing process. Indirectly, MSC could also release biological factors, which will be able to stimulate the resident disc cells or activate the potential progenitor cells present within the IVD. Therefore, stem cell homing into damaged tissues may be seen as a promising therapeutic method for tissue regeneration. Consequently, we have investigated potential of these hyaluronan-based hydrogels as a carrier for chemokines, to induce stem cell homing within the degenerated IVD. We will present our recent ex vivo and in vivo results on the delivery of SDF-1 and CCL5 using the above hydrogels in bovine and ovine intervertebral discs.

PROF. L. AMBROSIO (ITALY)

Director of Chemical Sciences & Materials Technology Department, National Research Council of Italy.

Prof. Luigi Ambrosio received the doctoral degree in Chemical Engineering (1982) from University of Naples "Federico II". He was Research Associate at University of Naples (1983-1985), Research Associate at University of Connecticut, USA (1985-1986), and Visiting Scientist at Kontron Medical Inc., USA (1986- 1988). Adjunct Professor of University of Connecticut, USA (1997-2003) and of University of Naples "Federico II" (1997-2010). Director of Institute of Composites and Biomedical Materials, National Research Council of Italy. (2008-2012). Co-Director of "Multifunctional Polymers & Biomaterials Research Centre", Sichuan University-CNR, Chengdu, China (since 2013). He is member of Advisory Board and Guest Editor of International and National Scientific Journals, Vice-President of the Italian Society of Biomaterials (2006-2013), and President of the European Society for Biomaterials (2006-2013), Past President since 2013. He has been nominated Fellow of the American Institute for Medical and Biological Engineering (March 2001), and Fellow of Biomaterials Science and Engineering (May 2004). He received the APA Distinguished Award (2014) and the European Society for Biomaterials G. Winter Award (2015). Member of the European



Commission Advisory Group of the FP7-NMP (2006-2008) and Member of High Level Group - Key Enabling Technologies- European Commission (2009-2015). Research interests include design and characterisation of polymers and composites for medical applications and tissue engineering, rheology of biological fluids, structural properties of natural tissue, properties and processing of polymers and composites and nanostructures, hydrogels and biodegradable polymers. Publications include over 300 papers on international scientific journals and book, 18 patents, more than 400 presentations at international and national conferences and over 120 invited lectures.

ADVANCED STRUCTURES AND TECHNOLOGIES FOR TISSUE REGENERATION

In biomedical applications, the capability of controlling physical and chemical interactions at the level of elementary natural components, from proteins to cells, is necessary to offer a more efficient investigation, manipulation, and application of living systems. Micro or nano-structured polymers in the form of nanoparticles, nano-fibers and nano-composites have gained increasing interest in regenerative medicine because they are able to mimic the physical features of natural extracellular matrix (ECM) at the sub-micro and nano-scale level. By their manipulation via nanotechnologies, they currently represent an interesting tool to reproduce the features of native cell microenvironment, which determine tissue specificity and architecture of tissues. In this context, many nano-inspired processes and tools have been recently studied to acquire better knowledge on the natural evolution of healthy or pathological tissues in 3D scaffolds, and to discover new technological solutions to improve conventional strategies in tissue engineering. By a careful selection of materials and processing conditions, they offer the opportunity to finely control characteristic shapes and sizes from micro to sub-micrometric scale and to incorporate biopolymers and bioactive molecules such as proteins or growth factor. Design of different nanostructures are made by electrospinning technique, rapid prototyping and biomineralization process, is proposed to develop active platforms to support the regeneration of different tissues such bone, IVD and nerve.

PROF. S. BARINOV (RUSSIAN FEDERATION)

PhD and Dr Sci (materials science), professor, correspondent member of RAS, head of the "Ceramic Composites" Laboratory of the Baikov Institute of Metallurgy and Materials Science Russian Academy of Sciences.

AN EXPERIENCE IN DEVELOPMENT OF CALCIUM PHOSPHATE MATERIALS: FROM BONE SUBSTITUTION TO BONE REGENERATION

Two fundamentally different approaches to the recovery of damaged bone functions are feasible, namely, the mechanical replacement of bone by an implant and the regeneration of the bone tissue. Correspondingly, two kinds of implant materials that differ in their microstructures, properties and in vivo behaviour in the human body were elaborated. The calcium phosphate materials and the composites on their base are known to be the most prospective as implant material being biocompatible and osteoconductive in the human body. In this lecture a brief overview of the results obtained at the Baikov Institute of Metallurgy and Materials Science RAS (Moscow, Russia) over the last decade are presented. Starting from the chemical synthesis routes of various calcium orthophosphates, particularly cation- and anion-substituted to improve their biological behavior, the authors report the technological principles how to reach the high-strength level state of ceramics and how to design a porous structure of composites intended to serve as resorbable scaffolds in bone tissue engineering. A special attention will be taken on the additive technologies for preparation of scaffolds. Physical and chemical problems which can be taken into account for proper fabrication of the composites "biopolymer – calcium phosphates" are discussed as well.

PROF. S.M. BEST (UK)

Department of Materials Science and Metallurgy, Cambridge Centre of Excellence, University of Cambridge

Serena Best is a Professor of Materials Science and Fellow of St. John's College, Cambridge. She co-directs the Cambridge Centre for Medical Materials. She has published around 250 journal papers, books and book chapters and holds 9 patents in the fields of biomaterials and skeletal repair. She is a Fellow of the Royal Academy of Engineering and also the Institute of Materials, Minerals and Mining. She is an Editor of the Journal of Materials Science: Materials in Medicine and has been invited to act as a specialist on both national and international assessment panels.



PRODUCTION AND CHARACTERIZATION OF COLLAGEN-BASED SCAFFOLDS FOR SOFT TISSUE REPAIR

There have been phenomenal advances in the field of Tissue Engineering during the past 20 years. The simple ideas of tissue repair- have been replaced with cell-mediated tissue reconstruction and regeneration, and there is an increasing need for carefully designed scaffolds to deliver these cells to sites of disease or injury. The importance of pore structure and morphology have begun to be understood for soft tissue applications, but the nature of the pore interconnections is not always considered. We have investigated the physics behind production of lyophilised porous collagen scaffolds through ice crystal formation. By careful control of the mould design and processing conditions, a range of pore morphologies can be produced ranging from equiaxed to elongated and we have characterized the effects of these on cell migration. Scaffold composition is another critical consideration and there is a fine balance required between scaffold “activity” and mechanical performance. In collagen scaffolds in particular, control of the degree of crosslinking is essential to optimise,

simultaneously, for both mechanical and biological performance. This talk will cover the recent work that has been undertaken to optimise the structure and properties of scaffolds for a range of clinical applications in soft tissue repair.

PROF. A. BOCCACCINI (GERMANY)

Head of Institute of Biomaterials, Friedrich-Alexander University of Erlangen-Nuernberg, Editor Materials Letters

Aldo R. Boccaccini is Professor of Biomaterials and Head of the Institute of Biomaterials at the University of Erlangen-Nuremberg, Germany. He is also visiting Professor at Imperial College London, UK. He holds an engineering degree from Instituto Balseiro (Argentina), Dr-Ing. from RWTH Aachen University (Germany) and Habilitation from TU Ilmenau (Germany). The research activities of Prof. Boccaccini are in the field of glasses, ceramics and composites for biomedical, functional and/or structural applications. He is the author or co-author of more than 600 scientific papers and 15 book chapters. His work has been cited more than 14,000 times and he was named in the 2014 Thomson Reuters Highly Cited Researcher list. Boccaccini has been a visiting professor at different universities around the world, including Japan, Italy, Spain, Slovenia, Netherlands, Singapore, Germany, Argentina and Poland. His achievements have been recognized with several awards including, most recently, the Materials Prize of the



German Materials Society (DGM) in 2015. Boccaccini is the editor-in-chief of the journal *Materials Letters* and serves in the editorial board of more than 10 international journals. In 2015 he was elected member of the Council of the European Society for Biomaterials (ESB). He also serves in the Review Panel of the German Science Foundation (DFG) and is an international advisor to the Ministry of Science and Technology of Argentina.

SOFT TISSUE REGENERATION WITH BIOACTIVE GLASSES: EMERGING EVIDENCES AND SUCCESSFUL OUTCOMES

Biochemical reactions occurring at the interface between bioactive glasses (BGs) and biological environments, specially related to the release of BG dissolution products at

the glass–tissue interface, are relevant for both hard and soft tissue regeneration. In this presentation, the development of ion doped BGs for soft tissue engineering will be discussed, comprehensively covering current research in the field and considering silicate, phosphate and borate BG compositions. Selected in vitro and in vivo evidences that demonstrate the suitability of BGs for soft tissue repair will be discussed, including studies investigating vascularization, wound healing and nerve tissue repair [1]. Specific in-vitro studies which show the effects of BG dissolution products on cell behaviour in relation to angiogenesis will be discussed in detail considering novel BG compositions incorporating selected ions such as Co, Cu, Li and B. In addition, in-vivo investigations that assess vascularisation of BG scaffolds will be presented, which highlight the relevance of the angiogenic potential of BGs of different compositions, the effect of dissolution products on vascular growth factor release, induction of hypoxia conditions and endothelial cell behavior. Potentially active mechanisms of interaction of BGs and soft tissues based on the surface bioreactivity of BGs will be presented and the development of specific combinations of BGs and biopolymers to create flexible bioactive composites for soft tissue repair will be highlighted.

PROF. G. CIOFANI (ITALY)

Italian Institute of Technology Centre for Micro-BioRobotics, Polytechnic University of Torino

PIEZOELECTRIC NANOMATERIALS FOR TISSUE ENGINEERING

Nanoscale structures and materials have been explored in many biological applications because of their novel and impressive physical and chemical properties, that offer remarkable opportunities to study and interact with complex biological processes. In this talk, piezoelectric nanomaterials and their applications in the nanomedicine field will be introduced, with particular attention to tissue engineering and

regenerative medicine. Despite their impressive potentials, in fact, this kind of nanostructures have not yet received significant attention for bio-applications. Our results suggest that the exploitation of piezoelectric nanoparticles in nanomedicine is possible and realistic, and their impressive physical properties can be most useful for several applications, that range from sensors and transducers for the detection of biomolecules, to “sensible” substrates for tissue engineering or cell stimulation. After a short introduction to the major classes of innovative nanoparticles that have gained attention in the recent years, attention will be focused on the research carried out in our laboratories, introducing barium titanate nanoparticles, boron nitride nanotubes and polymeric composites based on these nanomaterials.



PROF. M. CULHA (TURKEY)

Department of Genetics and Bioengineering, Yeditepe University, Ataşehir, Istanbul

Prof. Dr. Mustafa Culha obtained his Ph.D. degree in chemistry from the University of Tennessee-Knoxville in 2002. Then, he worked as a post-doctoral researcher at Oak Ridge National Laboratory (2002-2003) before joining to Schering-Plough Corporation, NJ as an investigator. In 2004, he joined to the Genetics and Bioengineering Department of Yeditepe University. The utility of surface-enhanced Raman scattering (SERS) to develop novel detection and diagnostic tools for medical and biomedical applications, and understanding nanomaterial-living interactions to develop novel approaches for delivery and therapeutic applications are ongoing research projects in his laboratories. He and his colleagues have authored of more than 80 papers in prestigious journals, several book chapters and patents in the area of analytical and bioanalytical chemistry, and nanotechnology. He served as a panelist in several national and international panels such as H2020, NIH, FP7, German, Polish, Swiss



and Czech Science Foundations and TÜBİTAK. He is the editor of a special issue for Surface-enhanced Raman Scattering of Journal of Nanotechnology, a Nano-Bio special issue for Nanobiotechnology of Journal of Nanoparticle Research, and NanoSpectroscopy special issue of Analytical and Bioanalytical Chemistry. He is also on the editorial board of Applied Spectroscopy.

SYNTHESIS OF BORON NITRIDE NANOTUBES AND THEIR BIOMEDICAL APPLICATIONS

Boron nitride nanotubes (BNNTs) known as the structural analogues of carbon nanotubes (CNTs) are claimed to have superior properties than CNTs referring to their resistance to high temperature and harsh chemical conditions, high hydrogen storage capacity and electronic properties. Several synthesis methods such as arc-

discharge, chemical vapor deposition (CVD) or laser ablation using amorphous boron, boric acid, borazine or CNTs as starting materials were reported in recent years. However, all the approaches have some drawbacks such as uniformity, high cost and low yield. In our laboratories, the BNNTs were synthesized starting from one commodity boron compound, colemanite, in the presence of ammonium gas and iron (III) oxide catalyst at relatively lower temperatures. The synthesized BNNTs were thoroughly characterized with imaging and spectroscopic techniques. The synthesized BNNTs were investigated for their use in medical and biomedical applications such as gene and drug delivery, and tissue scaffolds after their toxicity evaluations. The results show that these nontoxic materials can be good candidates for novel medical and biomedical applications. The financial support from TUBITAK (Project no: 112M480) and Yeditepe University is gratefully acknowledged.

DR. E. GENTLEMAN (UK)

King's College London, Dental Institute, Craniofacial Development & Stem Cell Biology Division

Dr Eileen Gentleman is a Wellcome Trust Research Career Development Fellow in the division of Craniofacial Development & Stem Cell Biology at King's College London. She joined Imperial College London in 2005 as post-doctoral research associate (Stevens Group) after completing her PhD in Biomedical Engineering (Tulane University, USA). In 2011, she moved to King's where her research focuses on engineering the 3D cell niche to control stem cell differentiation for tissue engineering, particularly to regenerate osteochondral tissue. Her multi-disciplinary research interests also include fundamental mechanisms of biomineralisation and the role of mechano-sensing in tissue development. She has also worked extensively with bioactive glasses, and is interested in the biological effects of surface free energy and ion release on stem cell differentiation. Her work has been published in prestigious journals including *Nature Materials*, *Proceedings of the National Academy of Sciences USA*, and *Biomaterials*. Dr Gentleman has received



funding awards from the Wellcome Trust, the Rosetrees Trust, the Royal Society and Orthopaedic Research UK, and is a recipient of both a Wellcome Image Award (2016) and an MIT Koch Institute Image Award (2016). The Orthopaedic Research Society named her as a finalist for their New Investigator Recognition Award (2010) and in 2013 her work in regenerative medicine was recognised with a prestigious Philip Leverhulme Prize.

ENGINEERING THE STEM CELL NICHE FOR REGENERATIVE MEDICINE

The maturation of the field of regenerative medicine over the last decades has been accompanied by a renewed recognition of the role of the cell niche in directing appropriate stem cell differentiation and tissue formation. In short, a myriad of factors in a cell's local microenvironment direct its response, and so a new generation of

biomaterials engineered to mimic the niche and actively control cell behaviour are needed to develop clinically successful therapies. The cell niche is influenced by a complex combination of soluble mediators including growth factors, secreted factors, environmental factors, inflammatory mediators and even simple ions. However, there has also been a recent surge in our understanding of the importance of insoluble factors in directing cell response. These include the biological, chemical and physical properties of the extracellular matrix, including its stiffness, topography and composition. My group focuses on understanding these interactions and engineering materials that mimic the 3D cell niche so that we can direct cell response for regenerative medicine. Here I will talk about some of the avenues of research my lab explores, including using unsupervised techniques to analyse cell response to strontium-substituted bioactive glasses and the bi-directional interactions that take place between a cell and its niche when encapsulated in modifiable hyaluronic acid-based hydrogels.

DR. C. KRAFFT (GERMANY)

Leader of Research group "Optical Cell Diagnostics", Leibniz Institute of Photonic Technologies

Christoph Krafft is research group leader at the Leibniz Institute of Photonic Technology in Jena, Germany, and teaches physical chemistry at the Friedrich-Schiller-University Jena, Germany. He holds a physics degree from University Oldenburg (Germany), Dr. rer. nat. in biophysics from Humboldt University Berlin (Germany), and habilitation in analytical chemistry from University of Technology Dresden (Germany). The research activities of Dr. Krafft since 1994 are in the field of Raman and infrared spectroscopy of biomolecules, tissues and cells which includes microscopy, imaging and fiber optic technologies. He is the author and co-author of more than 100 peer-reviewed papers and 14 book chapters. His work has been cited more than 3,000 times (H index 33). He spent three years for postdoctoral studies at the University of Missouri Kansas City (USA) and University Trieste (Italy). He serves in the editorial board of the Journal of Spectroscopy and the Jour-



nal Biomedical Spectroscopy and Imaging, and was guest editor for the Journal of Biophotonics and Analytical and Bioanalytical Spectroscopy. He leads the working group "Histopathology of Cells, Tissue Sections and Biopsies from Cancerous and Noncancerous Pathologies" within the COST network Raman4Clinics.

OVERVIEW AND PERSPECTIVES OF RAMAN SPECTROSCOPY FOR DIAGNOSTICS OF CELLS AND TISSUES

Raman spectroscopy is an optical method to probe intrinsic molecular vibrations that provide a specific fingerprint of samples. Among the main advantages are that Raman spectra can be collected without labels and without destructing samples that is particular important for in vivo biomedical studies of cells and tissues. In this presentation, Raman spectroscopic applications and instrumentation will be

summarized to characterize and classify biomedical specimens. Near infrared lasers with 785 nm emission are popular excitation sources because autofluorescence is minimized and penetration of radiation is maximized. Confocal microscopes offer diffraction-limited lateral resolution which enables visualization of subcellular details. This has been used to collect Raman images and monitor the uptake of lipids in macrophages. A single Raman spectrum per cell is sufficient to classify the cell type. This principle is a promising approach to identify circulating tumor cells in blood of cancer patients. The limited throughput of Raman-based identification of rare cells can be overcome by pre-enrichment procedures. Raman imaging is also a complementary tool for the histopathological assessment of tissue sections. Label-free Raman images reveal information about the tissue morphology such as cell nuclei or tumor margins, and the tissue biochemistry such as nucleic acid, protein and lipid content. These parameters were shown to correlate with the malignancy of primary brain tumors. The molecular fingerprint of Raman spectra also allows determining the primary tumor of secondary brain tumors, i.e. brain metastases. Coherent Raman scattering reduces image acquisition from hours to seconds when coupled to laser scanning microscopes. Second-harmonic generation and two-photon excited fluorescence images can simultaneously be registered and give multimodal tissue contrast. Finally, Raman systems are connected to fiber optic probes for combination with endoscopy and intraoperative diagnostics. A proof-of-concept study demonstrated the detection of atherosclerotic plaques in living rabbits.

PROF. S. MACNEIL (UK)

Department of Materials Science and Engineering, University of Sheffield

Sheila MacNeil is Professor of Tissue Engineering in the Department of Materials Science and Engineering and the academic lead for the large, multidisciplinary Biomaterials and Tissue Engineering Group based within the Kroto Research Facility. Her research focuses on the development of tissue engineered skin and other epithelial tissues (oral mucosa, oesophagus, bladder, pelvic floor and cornea) and has successfully developed tissue engineered skin products through to commercialisation (MySkin™ and Cryoskin™) to benefit patients with severe burns and chronic wounds. Recent tissue engineering challenges include development of scaffolds for repair of the female pelvic floor, biomaterials to deliver corneal cells to patients and development of hybrid biomaterials for cleft palate repair. She has a strong interest in interdisciplinary research and seeks to translate clinical benefit to patients. She works closely with colleagues in the Faculties of Engineering, Science and Medicine, and also with clinical



colleagues in Burns, Dermatology and Urology. She is working with colleagues in India to develop an alternative to the amniotic membrane for transplantation of corneal limbal stem cells for patients with scarring of the cornea. In 2011 she established the cross faculty degree, Bio-engineering which involves 6 of the 7 departments of the Engineering Faculty and Medical Physics in the Medical School.

WHY WE LOVE 3D TISSUE ENGINEERED SKIN MODELS

Some of the more interesting and challenging clinical conditions are ones which you can't investigate adequately in 2D cell culture or in patients, or even in animal models as they often don't exist. For example it's very difficult to look at skin contraction in animal models and many aspects of pigmentation are difficult to explore. Also it's now well recognised that the complexities of the relationships between cells in 3D cannot

be adequately mimicked in 2D culture.

My laboratory, while developing tissue engineered skin for the treatment of burns patients, has produced a range of 3D human skin models which we have used to examine cell-cell communication and cell-extracellular matrix communication. To-date we have used these models to look at wound healing, skin contraction, bacterial infection, skin pigmentation , melanoma invasion and angiogenesis . We have also used them as platforms for developing tissue imaging technologies spanning confocal microscopy combined with novel fluorescent dyes to indicate hypoxia, non-invasive optical coherence tomography and Raman spectroscopy.

PROF. E. MELE (UK)

Department of Materials, Loughborough University, Leicestershire

Dr Mele is currently Senior Lecturer in Biomaterials at the Department of Materials of Loughborough University (United Kingdom). Her research interests include: Biocompatible and natural polymers for regenerative medicine; Nanofibrous wound dressings with antimicrobial activity and enhanced cell proliferation; Functional nanocomposites with controlled superficial and mechanical properties; Microfluidic devices for biological assays and food safety; Nanofabrication approaches for polymers.



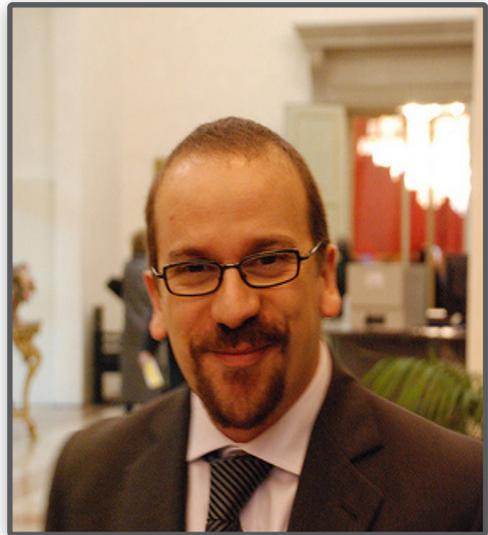
NANOFIBROUS ARCHITECTURES FOR REGENERATIVE MEDICINE

Physical or chemical traumas are often responsible for injuries of the human skin, with the consequent disruption of its normal physiology and the initiation of specific healing processes to restore the tissue functions. Acute or chronic wounds affect millions of people annually, and their incidence is expected to increase in the next years mainly due to the growth and aging of global population. Therefore, advances in wound care medical devices are required. Here we present the realization of composite electrospun nanofibers based on natural polymers (alginate, chitosan, silk and cellulose) as ideal candidates for drug delivery and wound dressing systems. In fact, the ultrafine size of the fibres guarantees excellent conformability of the non-woven mat to the wound site, proving complete coverage of the injured tissue, and protection against infections and dehydration. Moreover, the high porosity of the electrospun mesh facilitates the transport of nutrients and the absorption of exudates, together with the effective delivery of therapeutic substances.

PROF. L. MORONI (NETHERLANDS)

MERLN Institute for Technology-Inspired Regenerative Medicine, Complex Tissue Regeneration department, Maastricht University

Dr. Lorenzo Moroni studied Biomedical Engineering at Polytechnic University of Milan, Italy, and Nanoscale Sciences at Chalmers Technical University, Sweden. He received his Ph.D. cum laude in 2006 at University of Twente on 3D scaffolds for osteochondral regeneration, for which he was awarded the European doctorate award in Biomaterials and Tissue Engineering from the European Society of Biomaterials (ESB). In 2007, he worked at Johns Hopkins University as a post-doctoral fellow in the Elisseff lab, focusing on hydrogels and stem cells. In 2008, he was appointed the R&D director of the Musculoskeletal Tissue Bank of Rizzoli Orthopedic Institute, where he investigated the use of stem cells from alternative sources for cell banking, and the development of novel bioactive scaffolds for skeletal regeneration. From 2009 till 2014, he joined again University of Twente, where he worked as an assistant professor till 2013 and as an associate professor thereafter in the Tissue Regeneration department. Since 2014, he



holds an associate professor position at the MERLN Institute for Technology-Inspired Regenerative Medicine of Maastricht University. His research group interests aim at developing biofabrication technologies to generate libraries of 3D scaffolds able to control cell fate. In 2014, he received the prestigious Jean Leray award for outstanding young principal investigators from the ESB and the ERC starting grant

DESIGN OF SMART SCAFFOLDS FOR REGENERATIVE MEDICINE THROUGH BIOFABRICATION TECHNOLOGIES

A key factor in scaffold-based tissue and organ regeneration relies on enhancing (stem) cell-material interactions to obtain the same original functionality. Different

approaches include delivery of biological factors and surface topography modifications. Although both strategies have proved to augment cell activity on biomaterials, they are still characterized by limited control in space and time, which hampers the proper regeneration of complex tissues. Here, we present a few examples where the integration of biofabrication technology platforms allowed the generation of a new library of 3D scaffolds with tailored biological, physical, and chemical cues at the macro, micro, and nano scale. By engineering their topological properties, these porous biomaterials influence the activity of seeded cells, thereby initiating the regeneration of skeletal, vascular, and neural tissues. Future efforts should aim at further improving our understanding of scaffold topological properties to achieve a fine control on cell fate at multiple scales. This will enable the regeneration of complex tissues including vasculature and innervation, which will result in enhanced in vivo integration with surrounding tissues. By doing so, the gap from tissue to organ regeneration will be reduced, bringing regenerative medicine technologies closer to the clinics.

PROF. F. RUSTICHELLI (ITALY)

Polytechnic University of Marche, Ancona (Italy)

Prof. Franco Rustichelli studied Physics at SCUOLA NORMALE SUPERIORE di Pisa and is Professor of Physics at the Polytechnic University of Marche (Italy) and is Guest Professor at the University of Krakow (Poland) and at the University Steel and Alloys of Moscow (Russia). He worked for the EUROPEAN ATOMIC ENERGY COMMISSION for about 20 years. He has a vast experience in fields like material science, biophysics, bio-materials and stem cells research. He is using for these investigations in addition to conventional laboratory techniques, like X-ray diffraction and electron microscopy and AFM, especially X-ray and neutron scattering techniques available at European large Scale Facilities like European Synchrotron Radiation Facility (ESRF) and High Flux Reactor of ILL, build in Grenoble (France). He published more than 300 papers on international journals in different fields. He is currently presenting invited talks at International Conference especially related to Nanoscience and Nanotechnology and to Stem Cell Research. He was/is member of several international scientific committee and member of editorial board of scientific journals. He was chosen to be an evaluator for European Projects, Soros Foun-



ation Projects and Projects of foreign countries. He was the scientific director during several years of an International Summer School on Advanced Material Science and Technology in Jesi (Ancona). He has taken part to more than 40 EU Projects, in some of them as coordinator or task coordinator, exploiting his experience in experimental investigations using small angle scattering (SANS/SAXS), microtomography and other techniques, available at European Large Scale Facilities, for microstructural studies of materials for different applications in several industrial and biomedical fields.

ADVANCED TOMOGRAPHIC TECHNIQUES BASED ON SYNCHROTRON

RADIATION IN REGENERATIVE NEUROLOGY, CARDIOLOGY AND DENTISTRY

A short introduction will be given of the standard X-Ray micromotography, which allows a good visualization at 3D level of biomaterials, mineralized tissues and of the spatial distribution of injected stem cells. However, this technique fails to finely discern soft tissues. This limitation is overcome by using a recently developed technique, the X-Ray holotomography, which is also able to visualize at 3D level the microvascular network, without any need of contrast agents. This technique expresses the maximum of its potentialities in conjunction with phase contrast radiography. Then the results will be presented of a neurological study in connection with attempts to repair muscle damage in Duchenne muscular dystrophy, by transplanting intra-arterially myogenic stem cells into the muscle itself. Several in vivo microtomographic visualizations were obtained for different times after injection in homozygous *scid/mdx* mice, by obtaining a quantitative information of the kinetics of diffusion of the stem cells in the muscular tissue. After that, an experiment will be presented related to the use of stem cells to repair infarcted hearts. The above mentioned imaging techniques were used to detect, with high resolution, the 3D spatial distribution of rat cardiac progenitor cells in ex vivo conditions, inside the rat infarcted rat early after injection. Moreover, X-Ray holotomography was used to investigate the structure of the bone regenerated, in a clinical experiment, in areas of mandibles with large bone defects, by deposition of mesenchymal stem cells derived from dental pulp. Finally, the perspectives will be considered connected with the use in dentistry of biodegradable nanofibers functionalised with blood derivatives.

PROF. R.W. SIEGEL (USA)

Materials Science and Engineering Director, Rensselaer Nanotechnology Centre and Rensselaer Polytechnic Institute, Troy, New York

Dr. Richard W. Siegel is the Robert W. Hunt Professor of Materials Science and Engineering at Rensselaer Polytechnic Institute, where he was Department Head (1995-2000) and founding Director of the Nanotechnology Center (2001-15). He was a member of the Nanotechnology Technical Advisory Group of the US President's Council of Advisors on Science and Technology (2003-09), chaired the WTEC worldwide study of nanotechnology science and technology (1996-98) for the US government that led in 2001 to the US National Nanotechnology Initiative, and served as the first chairman (1992-96) of the International Committee on Nanostructured Materials. Siegel is a founder and Director of Nanophase Technologies Corporation, a Fellow of the Materials Research Society (MRS)



and the American Institute of Medical and Biological Engineering (AIMBE), and was a Humboldt Senior Research Awardee in Germany and a RIKEN Eminent Scientist in Japan.

NANOSTRUCTURE-BIOMOLECULE INTERACTIONS: THE KEYS TO UNDERSTANDING NANOBIMATERIALS FOR HEALTHCARE

Developments over the past few decades worldwide have greatly increased our ability to synthesize and utilize nanoscale building blocks to create advanced materials and devices with novel properties and a wide range of functionalities. Among their novel properties, nanostructures have been shown to elicit more favorable and selective bio-molecular and cellular responses than surfaces at coarser length scales, owing to nanoscale-specific, biomolecule-nanostructure interactions. Understanding and using these interactions is enabling nanobiomaterials to create a platform for a new healthcare field, nanomedicine. Fundamental to developing a clear understanding

of these interactions, and their eventual control, however, is the comprehensive characterization of nanobio-conjugates. In order to characterize these conjugates at a variety of biomolecule loadings, and for precisely controlled nanostructures with both positive and negative surface curvatures, several model experiments have been carried out. The results of these studies will be presented and discussed in the broader contexts of enabling new biocompatible materials and developing nanomedicine for improved healthcare.

PROF. C. VITALE-BROVARONE (ITALY)

Politecnico di Torino

Applied Science and Technology Department

Associate professor in Materials Science and Technology at the Department of Applied Science and Technology, Politecnico di Torino. Her main research interest is in biomaterials for medical applications. Master Degree in Materials Engineering in 1997, PhD in Materials Engineering at Politecnico di Torino in 2001, she has won fellowships for research stays at the Ecole de Chimie de Montpellier in France and at the Lawrence Berkeley National Laboratory, California, USA. She has authored 110 papers on international journals, h-index 25.

She has coordinated the EU-funded projects (MATCH, BIORESS) and she has been Team leader for the EU project RESTORATION. At present, she is co-



ordinating the H2020 project MOZART and she is the Principal Investigator of the ERC-CoG-2015 grant BOOST.

MULTIFUNCTIONAL NANOMATERIALS FOR SMART RELEASE

The use of organized mesoporous materials in biomedical applications continues to gain interest due to their well-defined nanoporous structure offering them the ability to store and release different molecules based on their nanopore size and shape. They offer several advantages such as large incorporation of drugs, control on the drug release kinetics, excellent payload stability. In addition mesoporous inorganic materials are amenable to surface functionalization which can enable tuning of the hydrophilicity/hydrophobicity character, encapsulation of a variety of drugs, combination with other vehicles (e.g. polymer) but can also impart stimuli-responsive and/or targeting properties.

Mesoporous bioactive glasses (MBGs), which combine the textural parameters of ordered mesoporous matrices with the properties of conventional bioactive sol-gel

glasses, have received increasing attention as bone-tissue regeneration systems. The ambition is to impart other biological functions, including anti-bacterial activity, as well as stimulation of osteogenesis and angiogenesis, by incorporating therapeutic metallic elements and drugs. This talk will also introduce the H2020 project MOZART, which is targeted to the development and application of mesoporous materials in several biomedical applications.

PROF. T.J. WEBSTER (USA)

The Art W. Zafiropoulo Professor and Chair, Department of Chemical Engineering, Northeastern University, Boston

President, U.S. Society For Biomaterials Founding Editor, International Journal of Nanomedicine

THE NEXT GENERATION OF IMPLANTS: USING NANOMEDICINE WITHOUT DRUGS TO CONTROL CELL RESPONSES

Nanotechnology (or the use of materials with one dimension less than 100 nm) has been revolutionizing the medical device field for several decades due to the ability of nanomaterials to circulate longer in the blood stream, penetrating cells and tissues, simultaneously detecting and treating disease, and targeting select cellular receptors. However, so far, nanotechnology has proven not to be the panacea for eliminating cancer, cardiovascular diseases, osteoporosis, neural diseases, and many other diseases. Issues such as toxicity, efficacy, drug loading, cost, and lengthy FDA approval times have still proven to be significant obstacles. This presentation will summarize recent advances in developing improved medical devices for faster approval by focusing on not changing chemistries, but altering surface energy of existing FDA approved chemistries at the nanoscale. Such approaches have led to improved interactions with mammalian cells (such as bone, cartilage, vascular, neural, bladder, etc. cells) and decreased interactions with immune cells (such as monocytes, macrophages, etc.) to minimize nanoparticle clearance. Studies focusing not just on traditional biodegradable polymers but also metal oxides will be covered. Approaches which have combined treatment with diagnosis (i.e., theranostics) will also be emphasized for orthopedic, neural, and cancer treatment.



INVITED TALKS

MAGNETIC DOMAIN WALL TWEEZERS: A POWERFUL TOOL FOR MECHANOBIOLOGY INVESTIGATION AT CELLULAR AND SUBCELLULAR LEVEL

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Keywords: magnetic tweezers, magnetic nanoparticles, mechanobiology

INTRODUCTION

In vitro tests are of fundamental importance for investigating cell mechanisms in response to mechanical stimuli or the impact of the genotype on the cell mechanical properties [1]. In particular, the application of controlled forces to activate specific bio-pathways and investigate their effects, mimicking the role of the cellular environment, is becoming a prominent approach in the emerging field of mechanobiology [2]. Here, we present an on-chip device based on magnetic domain wall tweezers (DWTs), which allows for the application of finely controlled and localized forces on target living cells. Our platform represents an unique example of a versatile and non-invasive technology for studies on single cells, fully compatible with real time optical monitoring of the cell activity upon quantitative and localized mechanical stimulation.

RESULTS AND DISCUSSION

DWTs are based on magnetic Domain Walls (DWs) nucleation and displacement in ferromagnetic conduits. In properly designed nano- and microstructures, DWs exert an intense stray field gradient which allow for trapping and finely manipulating single superparamagnetic beads, when a low intensity external magnetic field (H_e) is applied

[3][4][5].

To this scope, micrometric $\text{Ni}_{80}\text{Fe}_{20}$ rings are patterned on a Si/SiO_2 chip where HeLa cells are afterwards cultured, and magnetic particles of 1 μm and 500 nm are manipulated via the application of a rotating $H_e = 300$ Oe. We show the possibility to trap and handle with high resolution (down to 100 nm) magnetic particles towards a target cell membrane, as illustrated in Figure 1. In this way, the trapped beads exert a magnetic force (F_m) on the cell (see Figure 2a) of the order of hundreds pN.

As shown in Figure 2b, such mechanical stimuli produce a sizable local deformation of the cellular membrane.

The membrane indentation observed during the experiments is precisely quantified by a cellular profile analysis (see Figure 3) and upon evaluation of the beads position within the magnetic field originated by the domain wall, the force applied during the experiments can be accurately quantified via micromagnetic simulations [6].

For example, considering the experimental data illustrated in Figure 2b and 3, the local membrane indentation is 2 μm and $F_m = 480$ pN.

Moreover, we demonstrate that our device is suitable for the manipulation of magnetic beads inside the cell cytoplasm. This allows to apply controlled mechanical stimuli on the cellular nucleus or to transport biomolecules with high resolution inside the cell, in a completely biocompatible way. At this purpose, 500 nm particles are microinjected in the cell cytoplasm using micrometric glass needles and precisely manipulated applying $H_e = 300$ Oe, as illustrated in the frames of Figure 4.

CONCLUSIONS

In conclusion, our magnetic structures patterned on-chip are exploited to implement a non-invasive manipulation method, fully biocompatible and integrated with conventional setups for advanced cell investigation at cellular and subcellular level. Furthermore, with this approach, a precise quantification of the applied forces can be performed via micromagnetic simulations, using as input parameters the actual bead position with respect to the DW in the conduit.

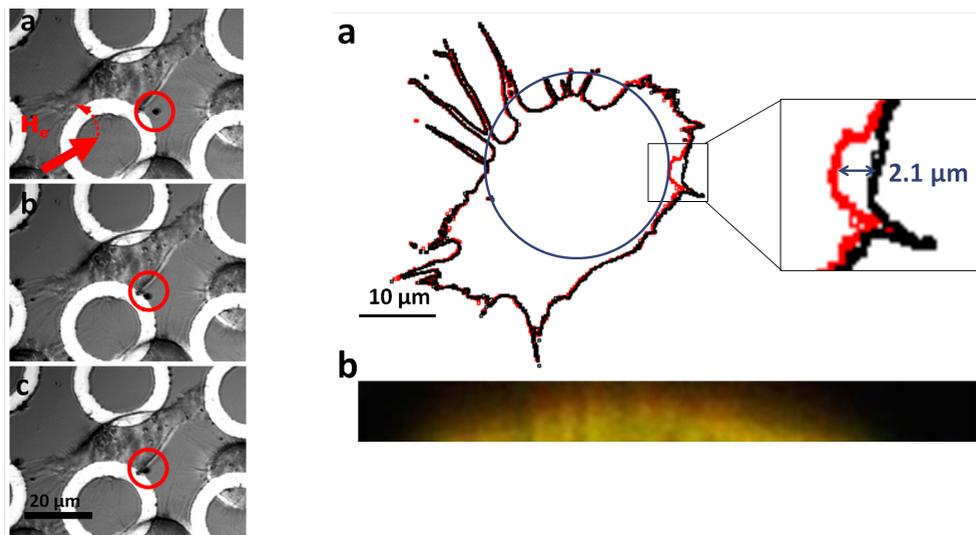


Figure 1: Frames from a video showing the attraction and manipulation of 1 μm particle to the cellular membrane of a target HeLa cell when a rotating $H_e=300$ Oe is applied

Figure 2: a) Sketch showing the device working principle: a superparamagnetic bead (blue) bound to a magnetic DW in the conduit exerts a magnetic force (F_m) on the cell membrane of a HeLa cell cultured on the chip surface. b) Optical image showing the HeLa cell membrane deformed by a cluster of 5 magnetic beads.

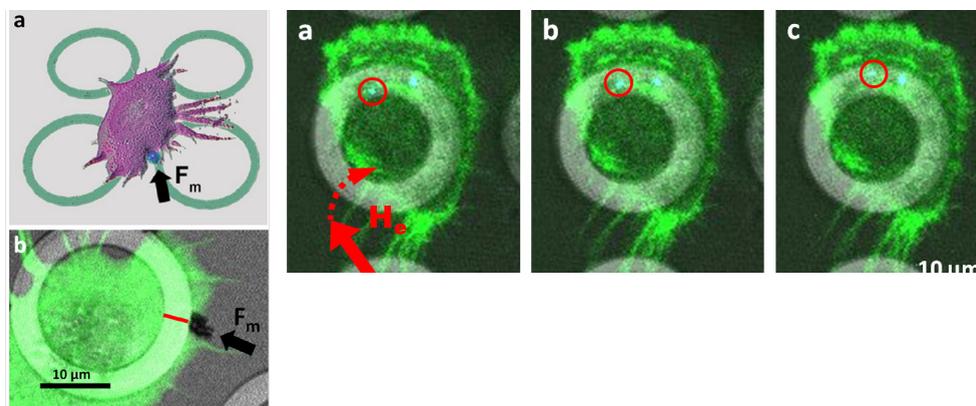


Figure 3: a) Overlapped cellular profiles before (black) and during (red) the local membrane deformation. b) A reconstruction of a HeLa cell slice in the plane perpendicular to the chip surface

before (orange) and during (green) the mechanical deformation.

Figure 4: Frames from a video showing the attraction and manipulation of a 500 nm particle functionalized with Cy3-dye (red fluorescence) inside the cellular cytoplasm of a target HeLa cell transfected with a F-actin marker, when a rotating He=300 Oe is applied.

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PIEZOELECTRIC NANOMATERIALS FOR TISSUE ENGINEERING

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Keywords: Piezoelectric nanomaterials; barium titanate nanoparticles; boron nitride nanotubes; tissue engineering

Nanoscale structures and materials have been explored in many biological applications because of their novel and impressive physical and chemical properties, that offer remarkable opportunities to study and interact with complex biological processes. In this talk, piezoelectric nanomaterials and their applications in the nanomedicine field will be introduced, with particular attention to tissue engineering and regenerative medicine. Despite their impressive potentials, in fact, this kind of nanostructures have not yet received significant attention for bio-applications. Our results suggest that the exploitation of piezoelectric nanoparticles in nanomedicine is possible and realistic, and their impressive physical properties can be most useful for several applications, that range from sensors and transducers for the detection of biomolecules, to “sensible” substrates for tissue engineering or cell stimulation. After a short introduction to the major classes of innovative piezoelectric nanoparticles that have gained interest in the recent years, attention will be focused on the research carried out in our laboratories (Figure 1), introducing barium titanate nanoparticles, boron nitride nanotubes, and polymeric composites based on these nanomaterials [1-3].

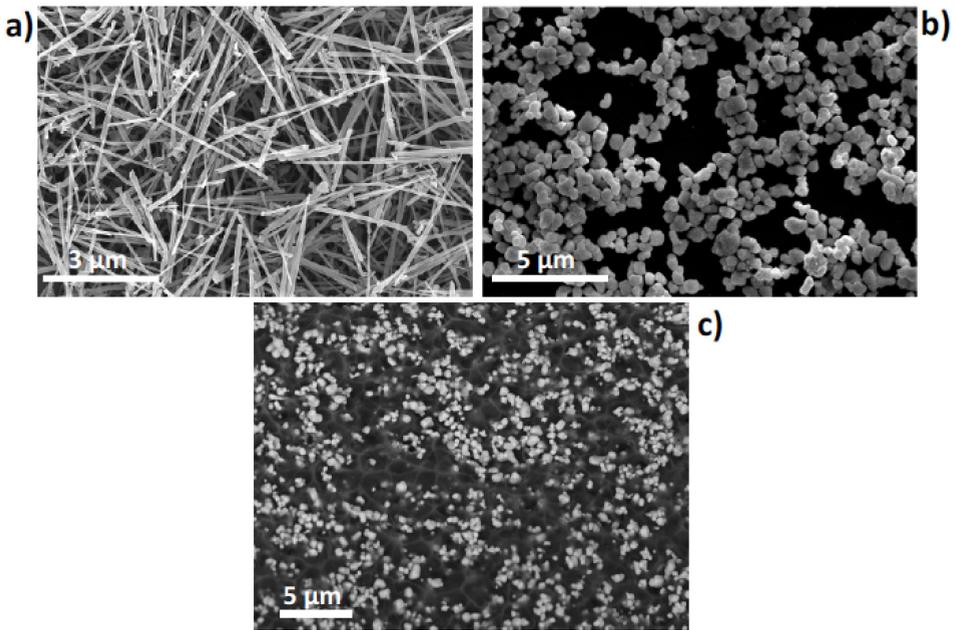


Figure 1: Scanning electron microscopy imaging of some piezoelectric nanomaterials: boron nitride nanotubes (a), barium titanate nanoparticles (b), and P(VDF-TrFE)/BaTiO₃ composites (c)

ACKNOWLEDGEMENTS

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SNOM SPECTROSCOPY FOR TISSUE IMAGING AND CANCER DIAGNOSTICS

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Keywords: Nanophonics, cancer diagnostics

INTRODUCTION

We present a fully implemented Infrared (IR) Scanning Near-field Optical Microscopy (SNOM) in spectroscopic mode for tissue imaging and early cancer diagnostics. The SNOM has been coupled with an infrared light source, based on Free Electron Laser at the ALICE facility in Daresbury.

RESULTS AND DISCUSSION

The potential of IR spectroscopy to characterise cancerous tissues has long been recognised and studies of various cancers by many groups have established that regions of malignant tissue can be easily identified on the basis of its IR spectrum. The oesophageal adenocarcinoma, the cancer with the fastest rise in incidence in the Western world, requires an instrument providing specific chemical images at sub-cellular level of oesophagus tissue. This approach demonstrates the potential of the IR-SNOM spectroscopy for yielding an accurate diagnostic test for oesophageal and other types of cancers.

The SNOM employed in this work was developed on the IR FEL at Vanderbilt and established on the IR FEL on the ALICE energy recovery linear accelerator at Daresbury [1]. Preliminary results of IR-SNOM on oesophageal adenocarcinoma have shown that the system can operate at nanometer resolution and has been able to distinguish between healthy and malignant tissues [2]. The optical fibre has been

driven in particular areas of the oesophageal tissue and topographical and optical images have been collected simultaneously at different wavelengths. In particular, SNOM images were collected at wavelengths of 7.0 mm (no strong biomarker), 7.3 mm (protein/glycoprotein), and 8.05 mm (DNA).

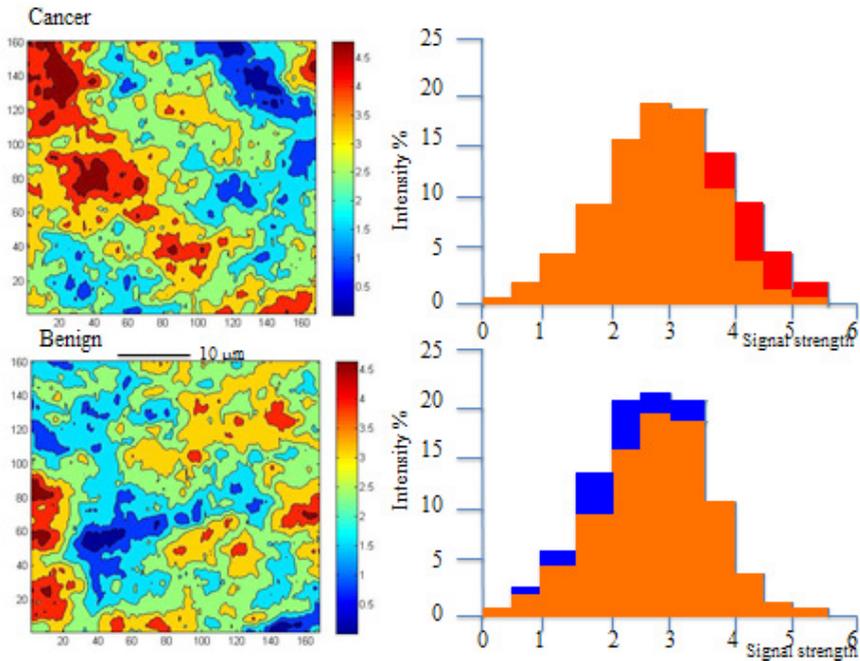


Figure 1: IR-SNOM image maps showing the location of intense DNA (red), intense protein/glycoprotein (blue) and of strong overlap of DNA and protein/glycoprotein (orange).

Figure 1 shows 40 mm × 40 mm optical SNOM images for two samples, labelled Cancer and Benign: the colour maps show the location of intense DNA (red), intense protein/glycoprotein (blue) and of strong overlap of DNA and protein/glycoprotein (orange). As clearly visible, Cancer sample shows a large spread of intense signal from DNA whereas Benign sample shows a lower overall density of DNA, which is more dispersed and exhibits more localised centres.

CONCLUSIONS AND OUTLOOK

This combination of InfraRed radiation and Scanning Near-field Optical Microscopy, in its spectroscopic mode, can be an important tool for tissue imaging and early cancer

diagnostics. It is expected to produce a major advance in imaging of malignant tissues [3], leading to the development of portable diagnostic devices for hospital use for various types of cancer. It is also planned to utilise the powerful combination of high spatial resolution and chemical specificity of the mentioned methodologies to study the key components, responsible for cancer formation.

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SYNTHESIS OF BORON NITRIDE NANOTUBES AND THEIR BIOMEDICAL APPLICATIONS

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Keywords: BNNTs synthesis, modification, toxicity, gene silencing, tissue engineering

INTRODUCTION

Boron nitride nanotubes (BNNTs) known as the structural analogues of carbon nanotubes (CNTs) are claimed to have superior properties than CNTs referring to their resistance to high temperature and harsh chemical conditions, high hydrogen storage capacity and electronic properties [1-6]. Several synthesis methods such as arc-discharge, chemical vapour deposition (CVD) or laser ablation using amorphous boron, boric acid, borazine or CNTs as starting materials were reported in recent years. However, all the approaches have some drawbacks such as uniformity, high cost and low yield. In our laboratories, the BNNTs were synthesized starting from one commodity boron compound, colemanite, in the presence of ammonium gas and iron (III) oxide (Fe_2O_3) catalyst at relatively lower temperatures. The synthesized BNNTs were thoroughly characterized with imaging techniques and spectroscopic techniques. The chemical functionalization of biomolecules including carbohydrates and oligonucleotides with BNNTs was accomplished through the $-\text{OH}$ groups on B atom at the edges or defects of the BNNTs. Covalent binding was formed between hydroxyl groups on BNNTs (h-BNNTs) and hydroxyl groups on bio macromolecules by cross-linking via glutaraldehyde. The synthesized BNNTs were investigated for their use in medical and biomedical applications such as gene delivery, and tissue scaffolds after their toxicity evaluations. The results show that these nontoxic materials can be good candidates for novel medical and biomedical applications.

RESULTS AND DISCUSSION

A range of reaction parameters such as temperature, heating time and type and amount of catalyst were optimized to improve BNNT synthesis efficiency. The formation of BNNTs was dramatically improved when Fe_2O_3 were used as catalysts compared to Al_2O_3 , FeCl_3 and Fe_3O_4 . The BNNTs were synthesized at a range of temperature and heating times from 500-1400 °C and times 30 min-150 min, respectively. The highest reaction yield were obtain at temperatures greater than 1100 °C and heating time of 60 min. The predetermined 32:1, 16:1, 12:1 and 8:1 colemanite:catalyst (w/w) ratios were tested and the highest yield was obtained at 12:1 ratio. In addition, the ball-milling applications were found to improve the reaction yield, enabling homogenous distribution of the reaction mixture and increasing the substrate surface area. Our studies shown that the synthesized BNNTs were localized the upper layers. The different boron sources such as B_2O_3 and H_3BO_3 were also evaluated but the yield of reactions was very low. The BNNTs were randomly oriented and multi-walled with an outer diameter of 10–30 nm and a wall thickness of 5 nm (Figure 1A).

For chemical functionalization, the formation of $-\text{OH}$ and $-\text{NH}_2$ functional groups at the edges or defects on BNNT structure is quite important as shown in Figure 1B since there must be a functional group on the BNNT surface for further covalent modifications.

While the pristine, h-BNNT and carbohydrate modified (m-BNNTs) did not show significant toxic effect on human dermal fibroblast (HDF) cells (Figure 1C), the BNNTs and h-BNNTs show toxic effect on A549 cancer cells in cytotoxicity, ROS detection and genotoxicity tests.

In gene silencing studies, MDA-MB-231-luc2 cells that express luciferase gene were used. The oligonucleotides complementary to the anti-sense morpholinos were covalently bound to the BNNTs (oligonucleotides-BNNTs). Morpholinos and their complementary oligonucleotides-BNNTs were hybridized in TAE buffer (Figure 1D). BNNTs-Morpholino structures were added to the cells and luciferase enzyme activity was measured. The reason why the activities of luciferase in which morpholino added into the cells decreased, silencing the gene by binding to the mRNA during the expression of morpholino's luciferase gene.

The tissue scaffolds were prepared with two approaches: freeze-drying by using chitosan polymer and electrospinning by using gelatin polymer. The inclusion of BNNTs and BNNT-OH in these scaffolds increased their mechanical strength and biocompatibility. Figure 1E shows that the improved cell proliferation and adhesion

onto the scaffolds can be attributed to the lower degradation rate of the structure that provides more stable surface for cells to proliferate and show their natural morphology.

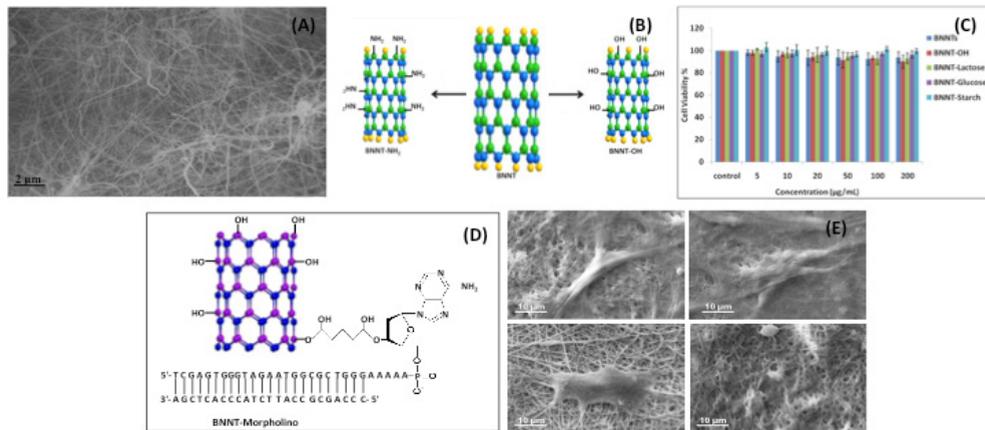


Figure 1: A) SEM image of synthesized pure BNNTs, B) BNNTs modifications, C) Cytotoxicity of BNNTs, D) BNNT-Morpholino, E) Cells onto the scaffolds including BNNTs.

CONCLUSIONS

In our laboratories, the BNNTs were synthesized from directly colemanite for the first time with CVD method by using Fe_2O_3 as a catalyst, 12:1 colemanite:catalyst ratio, under a NH_3 atmosphere at 1280°C , 135 min. Ca, Fe and other impurities in the obtained crude product were removed by two hours of washing with a 4 M HCl solution at 90°C to obtain pure BNNTs. The BNNTs were functionalized through the B atoms at the ends of the tubes and on the defects by formation of $-\text{OH}$ groups. The BNNT-OH was further covalently functionalized with carbohydrates and for possible medical and biomedical applications. The BNNTs and its derivate were found non-toxic to HDF cells but especially BNNTs and BNNT-OH toxic to A549 cancer cells indicating the induced cell death through increased reactive oxidative species. The toxicity results make it is a favourable nanomaterial for biomedical applications. In gene silencing studies, oligonucleotides complementary to the anti-sense morpholinos were covalently bound to the BNNTs and the morpholinos were hybridized to these BNNT-oligonucleotide structure. The BNNT-morpholino reduced the expression of the luciferase in the cells indicating the success of the approach. In

tissue engineering studies, the tissue scaffolds prepared with two approaches; freeze-drying and electrospinning, were compared with and without the addition of BNNTs and its derivatives. The scaffolds prepared with the inclusion of BNNTs decreased the decomposition rate, increased the mechanical strength and allowed the faster cell attachment on the scaffolds. In conclusion, the synthesis of BNNTs in yield and high quality with an economical approach at a reasonable temperature, chemical derivatization, and the application of its derivatives in medical and biomedical applications were demonstrated.

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[†]These authors have equally contributed to this work.

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BIOCERAMIC COATINGS WITH ANTIBACTERIAL PROPERTIES FOR INFECTION PROPHYLAXIS

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Keywords: bioceramic, antibacterial, thermal spraying

The demographical trend towards an aging population and the subsequent rise of osteoarthritis results in a growing demand for artificial implants every year. Various complications can be observed after bone replacement by prosthetic devices. Among the most important are mechanical problems such as aseptic loosening due to improper implant fixation, and surgical site infections due to bacteria colonization. While the first problem being well solved by depositing effective bioceramic coatings promoting osseointegration and firmly stabilizing the implant, the latter problem addresses new challenges in the field of biomedicine and technology.

A novel engineering approach is the development of calcium phosphate and bioglass coatings with incorporated fine dispersed metal species for infection prophylaxis. The bioceramic coatings enable the bone to grow firmly attached to the implant while the incorporated metal species act as infection prevention or antiseptic agents. Such an approach is easy to scale-up and is an important step forward in developing drug release coatings to address the most urgent problems in orthopaedics.

This presentation focuses on the development of such thin, multifunctional, antibacterial surfaces. The coatings were deposited by the innovative HVSF (High Velocity Suspension Flame Spraying) technology. With respect to the conventional powder-based thermal spraying technologies, HVSF is capable of yielding homogeneous and dense coatings with a reduced coating thickness. Antibacterial

properties were achieved by suspension functionalization with metals and metal salts (copper, silver and bismuth). Processing, microstructure, phase composition and biocompatibility were investigated.

ENGINEERING THE STEM CELL NICHE FOR REGENERATIVE MEDICINE

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Keywords: stem cell, hydrogel, regenerative medicine, bioactive glass

SUMMARY

The maturation of the field of regenerative medicine over the last decades has been accompanied by a renewed recognition of the role of the cell niche in directing appropriate stem cell differentiation and tissue formation. In short, a myriad of factors in a cell's local microenvironment direct its response, and so a new generation of biomaterials engineered to mimic the niche and actively control cell behaviour are needed to develop clinically successful therapies. The cell niche is influenced by a complex combination of soluble mediators including growth factors, secreted factors, environmental factors, inflammatory mediators and even simple ions. However, there has also been a recent surge in our understanding of the importance of insoluble factors in directing cell response. These include the biological, chemical and physical properties of the extracellular matrix, including its stiffness, topography and composition. My group focuses on understanding these interactions and engineering materials that mimic the 3D cell niche so that we can direct cell response for regenerative medicine. Here I will talk about some of the avenues of research my lab explores, including using unsupervised techniques to analyse cell response to strontium-substituted bioactive glasses and the bi-directional interactions that take place between a cell and its niche when encapsulated in modifiable hyaluronic acid-based hydrogels.

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OVERVIEW AND PERSPECTIVES OF RAMAN SPECTROSCOPY FOR DIAGNOSTICS OF CELLS AND TISSUES

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Keywords: Raman spectroscopy, coherent Raman scattering, surface enhanced Raman scattering, Microscopy, fibre optic probes

INTRODUCTION

Raman spectroscopy is an optical method to probe intrinsic molecular vibrations by inelastic scattering of monochromatic radiation that provide a specific fingerprint of samples. Among the main advantages are that Raman spectra can be collected without labels and without destructing samples that is particular important for in vivo biomedical studies of cells and tissues. In this presentation, Raman spectroscopic applications [1] and instrumentation [2] will be summarized to characterize and classify biomedical specimens.

INSTRUMENTATION

Main components of common Raman systems are lasers for excitation, optics for focussing the radiation on the sample, optics to collect the scattered radiation, a filter to suppress elastic scattered radiation, spectrograph to disperse the inelastic scattered Raman signals and CCD detector. Near infrared lasers with 785 nm emission are popular excitation sources because autofluorescence is minimized and penetration of radiation is maximized. Confocal microscopes offer diffraction-limited lateral resolution which enables visualization of subcellular details. A motorized stage move the sample in x-y-z dimension to collect Raman images.

RAMAN SPECTROSCOPY OF SINGLE CELLS

Figure 1 shows a series of Raman images monitoring the uptake of lipids in macrophages. Instead of staining by fluorescent dyes to highlight certain entities, careful analysis of the Raman data set is applied. The hyperspectral unmixing algorithm vertex component analysis (VCA) decomposes the data matrix into spectral contributions of nucleic acids, proteins and lipids. The chemical contrast displays cell nuclei with nucleoli (blue), cytoplasm (green) and lipid droplets (red). A single Raman spectrum per cell together with classification routines that were trained with well characterized samples is sufficient to represent the cell type and cell state (normal, stressed, apoptotic, necrotic...). This principle is a promising approach to identify circulating tumour cells in blood of cancer patients. The limited throughput of Raman-based identification of rare cells due to exposure time in the range of seconds per spectrum can be overcome by pre-enrichment procedures of a cell population.

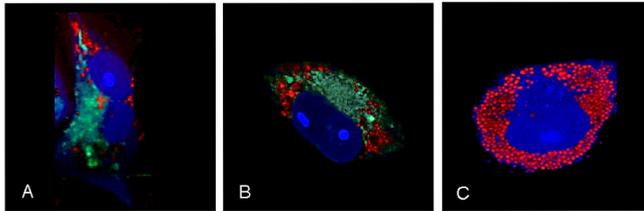


Figure 1: Raman images of macrophage cells in aqueous solution with subcellular resolution. Color codes represent different biomolecules such as lipids in droplets (red), nucleic acids in cell nuclei (blue) and proteins in cytoplasm (green) as obtained by hyperspectral unmixing. The amount of lipid droplets increase in the series of Raman images.

RAMAN SPECTROSCOPY OF TISSUE SECTIONS

Raman imaging is also a complementary tool for the histopathological assessment of tissue sections. Raman images in figure 2 show unstained tissue sections that were analysed by VCA. More biomolecules were identified than in figure 1. In principle, the data reveal information about the tissue morphology such as cell nuclei or tumor margins, and the tissue biochemistry such as nucleic acid, protein and lipid content. These parameters were shown to correlate with the malignancy of primary brain tumours. Once the correlation between Raman spectra and histopathology

is established and appropriate classification routines are trained, the molecular fingerprint of single Raman spectra also allows determining the tissue type and state (normal, inflammation, dysplasia, tumour...).

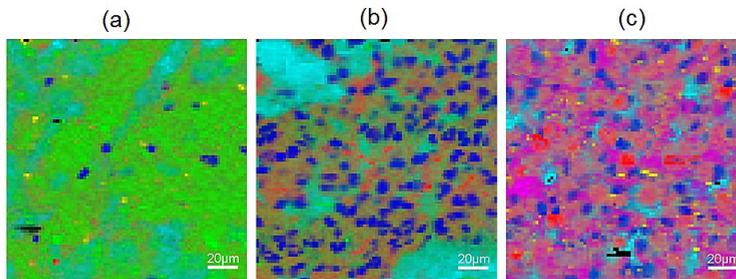


Figure 2: Raman images of brain tissue section in aqueous solution with cellular resolution. Color codes represent represent different biomolecules such as lipids (green), nucleic acids (blue), proteins (red), cholesterol ester (magenta) and buffer (cyan) as obtained by hyperspectral unmixing. Brain tissue with regular cell density is dominated by lipids (a), malignant brain tumours with high cell density contains low lipid content (b) and cholesterol ester is accumulated in necrotic tissue (c)

FIBER OPTIC RAMAN SPECTROSCOPY

Raman systems are connected to fiber optic probes for combination with endoscopy and intraoperative diagnostics. The required optics and filters are integrated into small diameter tubes. This assembly needs to withstand sterilization procedures. A proof-of-concept study demonstrated the detection of atherosclerotic plaques in living rabbits. The animals were fed by a high cholesterol diet to induce plaque deposits. The fiber optic probe integrated one central excitation fibre surrounded by 12 collection fibres that were arranged in a line along the spectrometer entrance slit. The position of the probe inside the rabbit was monitored by angiography (Figure 3). A Raman spectrum of plaque deposits in the aorta near the heart is typical for triacylglyceride lipids. Spectral contributions of surrounding blood and vessel wall were found to be weak at 785 nm excitation.

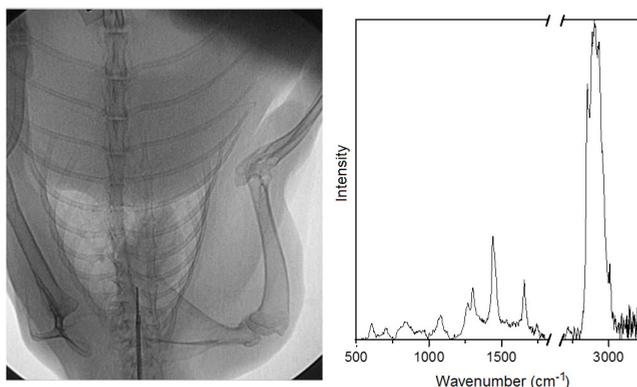


Figure 3: In vivo fiber probe Raman spectroscopy in rabbits. The position of the probe was monitored by angiography. Intense Raman signals of lipids were found in the arterial wall near the heart. The Raman spectrum is typical for triglyceride.

CONCLUSIONS AND/OR OUTLOOK

Raman spectroscopy is a versatile method to assess cells, tissues and biomaterials. Three examples were presented. Limitations occurred from relative weak signals from biomolecules and overlap with autofluorescence. Raman signals can be enhanced by coherent anti-stokes Raman scattering, resonance Raman scattering and surface enhanced Raman scattering at nanoscale gold or silver surfaces. Autofluorescence can be suppressed by smart data processing or selection of appropriate wavelengths. Standardization and translation of Raman-based techniques into the clinic is scope of the COST network Raman4clinics.

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HIGH PERFORMANCE POLYMERS USED FOR THE DENTAL IMPLANTS PROSTHETIC RESTORATIONS

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Keywords: High Performance Polymers, occlusal shock absorption, dental implant

INTRODUCTION

The lack of a periodontium for a dental implant may lead to high stress concentration at the bone level when the implants are prosthetically loaded. Several attempts were made over the years in order to mitigate the impact of occlusal forces to the implant support, absorb and dissipate them. Even from 1988 authors like Davis DM propose the use of resin materials for the implant supported prosthetic restorations veneering in order to mitigate the impact of occlusal forces [1]. Studies demonstrated that stress values in the bone depend on both the framework and veneer materials [2,3]. Today, new concepts are developing to resolve the problem of occlusal forces dissipation. The use of complex high performance polymeric materials is more and more frequently suggested usually for the realization of the prosthetic restoration substructure instead of the alloys, but also for the prosthetic abutment or even implant realization. The aim of this study is to review the current possibilities of using high performance polymeric materials for the implant supported prosthesis making in order to mitigate the impact of occlusal forces, discuss them in the light of the actual scientific knowledge and foresee the future tendencies in this highly current issue for the dental prosthetics.

HIGH PERFORMANCE POLYMERS AS VENEERING MATERIALS

An occlusal material with a low modulus of elasticity might dampen the occlusal impact forces, thereby decreasing its effect on the bone-implant interface. A range

of composite resins commercial products have been designed in order to use a property of these materials as through their modulus of elasticity to allow absorption of the energy generated during mastication [4]. Our experience sustain this idea and the veneering of implant supported restorations with a high-performance polymer represents today's method of choice in implant prosthetics techniques, but their role it is important in the case of full-arch fixed implant-supported prosthesis with cantilever distal extensions and less in single implant restorations.

HIGH PERFORMANCE POLYMERS AS FRAMEWORK MATERIALS

The spectacular development in recent years of the implant supported prosthetics has led to an intensification of the manufacturers researches in this direction with development of new materials from this range with occlusion shock absorbing abilities. Fiber-reinforced composites prostheses may be a good alternative compared with conventional metal framework implant-supported prosthesis in the future due to their biomechanical advantages [5].

Polyaryletherketones (PAEKs) are a group of high-performance semicrystalline thermoplastic resins, which family members differ according to their ratio of keto- and ether-groups. In medicine, PEEK (poly-ether-ether-ketone), the best-known PAEK member, mainly serves as implantation material due to its mentioned features and good biocompatibility. PEEK has an elasticity modulus similar to that of the bone [6]. Therefore, PEEK can be expected to absorb part of the forces generated during mastication and to limit their dissipation to the cervical area of the peri-implant bone. The PEEK frameworks can be constructed either via CAD/CAM manufacturing or via the conventional lost wax technique.

BioHPP® (High Performance Polymer) is a PEEK variant that has been specially optimised by the Bredent (Selden, Germany) for the dental field. This modified PEEK material is a biocompatible, nonallergic, rigid material, with flexibility comparable to bone, high polishing and low absorption properties, low plaque affinity, and good wear resistance [7]. Our experience with BioHPP lead us to good prosthetic results from a biological perspective but also aesthetically.

In the same category 3M ESPE (U.S.A.) also promotes a nano ceramic resin - Lava Ultimate Restorative. Another commercial products that intend to combine the advantageous properties of ceramics, such as durability and color stability, with those of composite resins, such as improved flexural properties is Cerasmart, a high-density composite resin material containing 71% filler particles by weight [8].

Polymers-infiltrated-ceramic-network (PICN) consist of two interlocking phases, a porous sinterized feldspathic ceramic and an infiltrating polymer (for dental use commonly methacrylates) The flexural strength, the elastic modulus similar to the tooth and lower hardness of these materials make PICN an option to be considered as prosthetic material [9].

OTHER USAGE POSSIBILITIES OF HIGH PERFORMANCE POLYMERIC MATERIALS IN IMPLANT PROSTHODONTICS

Some authors claimed that PEEK could represent an alternative biomaterial with its elastic modulus 3-4 GPa and indicated PEEK as being a substitute also for titanium-based implants, considering the radiographic, biomechanical, and histological results [10]. In addition, polymeric materials have gained greater popularity due to their high mechanical resilience and shock absorption properties. Considering the adequate biocompatibility, PEEK could prove to be a viable alternative to titanium in constructing implant abutments [11].

CONCLUSIONS

The impact reduction of occlusal forces to the implant support represents a current problem for the implant prosthodontics especially in the case of full-arch implant-supported fixed prostheses. If the use of resin materials with a lower modulus of elasticity for the implant supported prosthetic restorations veneering is a method already suggested from a while, in addition today we may also use materials based on high performance polymers like PEEK instead of the rigid non-precious metal alloys for the framework of these prostheses. Moreover, the biological and mechanical properties of these polymers may be used to develop more suitable materials also for the intraosseous implants and the prosthetic abutments in order to obtain prosthetic restorations with a better biological and functional integration.

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NANOFIBROUS ARCHITECTURES FOR REGENERATIVE MEDICINE

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Keywords: electrospinning, biomaterials, cell encapsulation, wound healing

INTRODUCTION

Physical or chemical traumas are often responsible for injuries of the human skin, with the consequent disruption of its normal physiology and the initiation of specific healing processes to restore the tissue functions. Acute or chronic wounds affect millions of people annually, and their incidence is expected to increase in the next years mainly due to the growth and aging of global population. Therefore, advances in wound care medical devices are required. Here we present the realization of composite electrospun nanofibres based on natural polymers (alginate, chitosan, silk and cellulose) as ideal candidates for drug delivery and wound dressing systems. In fact, the ultrafine size of the fibers guarantees excellent conformability of the non-woven mat to the wound site, proving complete coverage of the injured tissue, and protection against infections and dehydration. Moreover, the high porosity of the electrospun mesh facilitates the transport of nutrients and the absorption of exudates, together with the effective delivery of therapeutic substances.

RESULTS AND DISCUSSION

The biopolymers were dissolved in the appropriate solvent and extruded in form of nanofibers by applying an external electric field. Optimized amount of active agents, like essential oils with antibacterial activity, were incorporated inside the solution before the electrospinning process. The realized fibrous scaffolds were characterized in terms of morphology, mechanical and wetting properties, biocompatibility, drug

release and biodegradability.

We demonstrated the realization of ultrafine fibers of alginate and chitosan (diameter of about 50 nm) encapsulating active agents with antibacterial and healing activity [1]. We were able to control the degree of cross-linking of the composite fibers and, consequently, to regulate their degradation rate under physiological conditions, and the delivery over time of antimicrobial agents. Furthermore, we realized cellulose nanofibrous constructs with unique chemical and wetting properties by choosing the right combination of chemical agents and biopolymer [2-3]. Natural antimicrobial compounds, in particular essential oils, were incorporated into the nanofibers, in order to obtain controlled release of functional molecules. The fibers were characterized by antiseptic activity.

CONCLUSIONS

The produced composite and bioactive scaffolds can have a high potential impact on the next generation of biomedical devices for regenerative medicine.

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ADVANCED TOMOGRAPHIC TECHNIQUES BASED ON SYNCHROTRON RADIATION IN REGENERATIVE NEUROLOGY, CARDIOLOGY AND DENTISTRY

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Keywords: Neurology, Cardiology, Dentistry, Stem Cells, Imaging

INTRODUCTION

A short introduction will be given of the standard X-Ray micro computed tomography (micro-CT), which allows a good visualization at 3D level of biomaterials, mineralized tissues and of the spatial distribution of injected stem cells. However, this technique fails to finely discern soft tissues. This limitation is overcome by using a recently developed technique, the X-Ray holotomography, which is also able to visualize at 3D level the microvascular network, without any need of contrast agents. This technique expresses the maximum of its potentialities in conjunction with phase contrast radiography, especially in conjunction with thin/low absorbing systems. Actually, the X-Ray holotomography was applied for the first time to investigate a biological system by some of the authors of this presentation, together with other orthopaedists, by obtaining the visualisation at 3D level of blood vessels within a pore of a ceramic scaffold. The vessel network was connecting different areas inside the tissue-engineered bone, the growth of which was stimulated by stem cells deposited on the inner surface of pores of the ceramic scaffold [1]. Since then, other applications of holotomography were performed in the field of regenerative orthopaedics, but this subject will

deliberately not be considered in this presentation, which will be focused on applications in Regenerative Neurology, Cardiology and Dentistry.

RESULTS AND DISCUSSION

At first, the results of two neurological studies in connection with attempts to repair muscle damage in Duchenne muscular dystrophy, by injecting stem cells, will be presented. In both studies, X-Ray micro-CT was used in order to track their fate and in this way to obtain a better understanding of their role in the repair of damaged tissues produced by the disease.

In the first study [2], human stem cells derived by blood were labeled with nanoparticles of FeO and transplanted via intra-arterial infusion in a dystrophic animal model, namely scid/mdx mice. Ex vivo samples of 2 mm × 2 mm × 2 mm were investigated by X-ray micro-CT analysis in order to obtain the 3D spatial distribution of the injected human stem cells. It appeared that the cells were distributed around the vessels of muscle tissues within 24 hours of their intra-arterial transplantation.

The second neurological experiment [3] was performed on the same animal model affected by the same pathology, but in vivo conditions, at the European Synchrotron Radiation Facility in Grenoble, France. Several in vivo microtomographic visualizations were obtained for different times after injection by obtaining a quantitative information of the kinetics of diffusion of the stem cells in the muscular tissue. It appeared that a saturation effect in the diffusion process of stem cells occurs at maximum two hours after injection.

After that, an experiment related to the use of stem cells for repairing infarcted hearts will be presented [4]. The above mentioned imaging techniques were used to detect, with high resolution, the 3D spatial distribution of rat cardiac progenitor cells in ex vivo conditions, inside the rat infarcted heart, early after injection. It was possible to observe the presence of the stem cells inside the damaged cardiac tissue, with important structural details, which are difficult to obtain by conventional bi-dimensional images. Moreover, X-Ray holotomography was used to investigate the structure of the regenerated bone, in a clinical experiment, in areas of mandibles with large bone defects, by deposition of mesenchymal stem cells derived from dental pulp [5]. The 3D structure of regenerated bone in several patients, 3 years after implantation, was obtained, together with the blood vessel network.

Finally, the perspectives connected with the use of biodegradable nanofibers, functionalised with blood derivatives and/or products from activated thrombocytes

for accelerating the bone healing after a large extraction of teeth, in dentistry, will be considered. Functionalised nanofibers can be used in a form of a novel 3D nanofiber scaffold or, alternatively, as a nanofiber-based injectable controlled drug delivery system, in a form of a composite with self-assembling peptide gel.

CONCLUSIONS

The results which were obtained by the considered 3D imaging techniques, based on X-Ray synchrotron radiation, one of which (holotomography) very innovative and well suitable for investigating soft biological tissues, represent a very important progress as compared to the classical bi-dimensional histological techniques, which require a long and very tedious work for a 3D reconstruction, for instance, of the injected stem cells, in different areas of Regenerative Medicine. However, histology remains fundamental for a direct 2D observation of the biological details.

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NEW SMART, NATURE-INSPIRED BIOCERAMICS AND HYBRID COMPOSITES FOR BONE REGENERATION AND NANOMEDICINE

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Keywords: Biomimetic scaffolds, biomorphic transformation, biomineralization, hierarchical structure, hybrid composites

INTRODUCTION

The progressive ageing of the population and the extended life expectancy are increasingly pushing towards new smart solutions for regeneration of bone tissue diseased by trauma, tumours or degenerative pathologies strongly impacting on the life quality and the healthcare costs. In this respect, due to the different features of bone tissue in different anatomical regions, the design of bone scaffolds has to be specifically tailored on specific clinical applications. Calcium phosphates, particularly hydroxyapatite, are elective biomaterials for bone regeneration. However, the bioactive properties of biological-like hydroxyapatite nanophases are destroyed during the classical ceramic process, particularly the sintering. Therefore new approaches are needed for biomaterials synthesis and, in this respect, taking inspiration from nature is rapidly becoming a pivotal concept in material science, to take advantage of the outstanding properties and complexity of natural structures to develop new smart materials with unprecedented properties.

A new synthesis approach based on bio-inspiration was based on the reproduction of the biological processes yielding formation of new bone tissue in vivo (biomineralization). This approach intended to exploit the information stored in biologic macromolecules

such as collagen, to control the assembly and the simultaneous mineralization with nano-apatites presenting nearly amorphous crystal structure and multiple ion substitutions.

A very serious and still unmet clinical need is the regeneration of long, load-bearing bone segments; to this purpose bone scaffolds are required to exhibit complex morphological features and superior mechanical properties, associated to a biomimetic composition enabling fast bone formation and penetration and, possibly, progressive bio-resorption. In this respect, also due to the intrinsic limitations of the classical ceramic process, the achievement of such devices has been a serious challenge so far. Therefore, a revolutionary approach for bioceramic synthesis was ideated, by taking inspiration from natural structures such as woods. In particular, woods selected for their structure closely resembling the osteon structure of long bones were subjected to biomorphic transformations generating 3D scaffolds made of biomimetic, ion-substituted hydroxyapatite, with hierarchically organized structure reproducing the one of the starting wood.

An ever growing need in medicine is also to provide more effective and personalized therapies, to achieve enhanced safety and targeting to diseased tissues, without incurring in harmful effects due to systemic drug administration or to not effective delivery of relevant drugs in situ. In this respect, the use of magnetic fields in nanomedicine is an emerging concept that is today prevalently based on the use of cytotoxic iron oxide nanoparticles as superparamagnetic media. In this respect, a new biocompatible magnetic phase, i.e. a hydroxyapatite nanophase presenting controlled substitution with $\text{Fe}^{2+}/\text{Fe}^{3+}$ ions was recently developed and considered as a potential multifunctional platform for nanomedicine, characterized by enhanced safety and effectiveness.

RESULTS AND DISCUSSION

Bio-inspired synthesis of bone scaffolds was successfully achieved to generate hybrid porous scaffolds made of type I collagen matrix mineralized with hydroxyapatite nanophases strongly mimicking the composition of the biological apatite. The activation of the same control mechanisms acting during new bone formation enabled the 3D organization of the collagen fibrils and the heterogeneous nucleation of the inorganic phase in specific negative charged loci of the collagen molecule, that in vivo are specific for the apatite nucleation. Such control mechanisms also constrained the newly formed inorganic phase into very small nuclei (i.e. few nanometers), as

occurring in the natural bone tissue [1]. The obtained mineralized hydrogels were stabilized by cross-linking, then the final scaffolds were obtained by directed freeze-drying techniques (Figure 1A). The scaffolds can be also obtained in the form of compositionally and morphologically graded 3D porous constructs mimicking the different tissues of osteochondral regions, thus obtaining, after freeze drying, monolithic scaffolds reproducing the subchondral bone, the mineralized cartilage and the hyaline cartilage [2]. The new hybrid scaffolds demonstrated excellent biologic performance and outstanding regenerative ability in small and large animal model[3], thus demonstrating that biomimetic features can opportunely instruct cells to activate and sustain the bone regenerative cascade.

The biomorphic transformations were successfully applied to generate 3D porous scaffolds with hierarchically-organized structures reproducing the one of the starting natural wood (Figure 1B). The transformation process did not make use of high temperature treatments for the final consolidation of the scaffold, as it was derived from directed transformation of 3D solid templates, therefore the final scaffolds exhibit biomimetic composition, i.e. an ionically-substituted hydroxyapatite characterized by nanosize grains, and outstanding mechanical performance, with strength much higher than sintered hydroxyapatite scaffolds. These new materials exhibited very good biologic performance by preliminary tests in small animal model. The channel-like porosity of these new scaffolds can also significantly improve the scaffold vascularization, that is one of the major issues in load-bearing bone regeneration [4]. The new Fe-doped apatite exhibited very good biocompatibility and intrinsic superparamagnetic properties (FeHA) which may open the way to new perspectives in the development of advanced therapies and diagnostic tools characterized by enhanced safety and effectiveness. Due to its properties, Fe-HA can be used as superparamagnetic nanoparticles replacing iron oxides, taking advantage by its ability to bind several bioactive molecules (Figure 1C) and intense hyperthermia properties, enabling controlled release mediated by weak alternated magnetic fields [5,6]. Also, FeHA can be internalized by cells and driven in a controlled and non-destructive manner to the targeted tissues; in this respect, FeHA demonstrated high effectiveness as contrast media for NMR-based analysis. FeHA can also be heterogeneously nucleated on self-assembling collagen matrices by biomineralization process, to give rise to hybrid superparamagnetic and biomimetic scaffolds with enhanced osteogenic character and ability to be remotely activated by low magnetic fields [7].

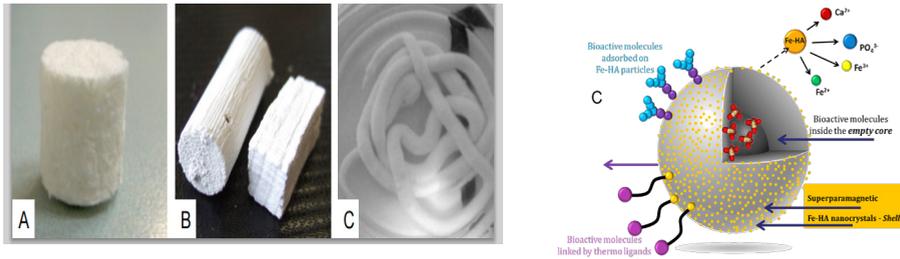


Figure 1: A: hybrid bone scaffold; B: hierarchically organized bone scaffold; C. scheme of FeHA nanoparticles functioning as drug linker.

CONCLUSIONS AND/OR OUTLOOK

The new scaffolds obtained by nature-inspired synthesis approaches exhibit very promising properties and outstanding regenerative ability, related to their intrinsic compositional and morphological features, thus demonstrating the relevance of high compositional and structural biomimesis as the source of cell-instructing signals to induce and sustain the bone regenerative cascade, thus preventing the use of biologics or growth factor. These new bio-devices can really solve very serious and still unmet clinical needs in orthopaedics, particularly the regeneration of entire osteochondral tissue complexes or of long, load-bearing bone segments, thus significantly reducing the recourse to repeated, costly and painful surgery and, also, completely recover the original biofunctionality and biomechanics. On the other hand, the new biocompatible and magnetic FeHA nanoparticles promise to represent a new safer and effective platform that can open new advanced applications in theranostics and regenerative medicine, with perspective of surpassing the use of the current superparamagnetic media that are however affected by long-term cytotoxicity.

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A DISULFIDE BOND-CONTAINING CELL SHEET ENGINEERING SYSTEM

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Keywords: Cell Sheet Engineering, polyvinylidene fluoride, oral mucosa, cornea epithelial cells

INTRODUCTION

Cell culture is one of the most basic skills of tissue engineering. The confluent cultivated cells were often treated with enzymes, but these treatments often lead to destruction of constructed extracellular matrix (ECM) during cell suspension formations. There limit the use and diminish the efficiency in animal study and also in applying cell therapy clinically. Therefore, establishing a system to enhance the bioavailability is very crucial. Okano et al. reported a system in which cell sheets were cultivated with on thermo-sensitive polymer and easily detached, pNIPAAm system [1], which greatly improves the outcome in many clinical studies significantly. Nevertheless, to improve the cell adhesion in a pNIPAAm system and reduce the toxicity of the residual NIPAAm monomer. Couple of years ago, we incorporate the idea by Akashi et al. which utilize disulfide bonds in forming the porous hydrogel [2], and further developed a non-pNIPAAm new way to culture and to detach cell sheet, which composed of disulfide bond-containing amino acid and biopolymers. So, we start attempting the new cell sheet engineering system to fabricate several type of cell sheets and manipulate them such as cornea epithelial cell, oral mucosal epithelial cell, periodontal ligament stem cell, dental pulp stem cell and endometrial stromal cell.

For example, the corneal epithelium is located in the outermost layer of visual pathway; it is also a part of the most often in contact or friction occurred with the outside world. In recent years, because of contact lenses with improper use, significant

increasing in the use of contact lenses population and age-related diseases, corneal epithelium injured patient is also significantly increased. Repair of corneal epithelium injury was mostly reliance on allograft as its solution till now. But the disadvantages of the lack of donor sources and immune response problems have been well known, it also can be said that today there is no a universal and effective autologous cornea epithelial tissue repair treatment. Cultivate few autologous tissue be a tissue-like cell sheet to reconstruct cornea epithelium is a pretty good way. Furthermore, women with endometriosis or experience of abortion surgery may suffer from endometrial stromal injury, and this injury can sometimes cause female infertility. In view of this situation, we studied the use of autologous endometrial cells as a source of cells for the reconstruction of endometrium. We utilized our system to engineer cell sheets as an alternative therapy of endometrial reconstruction. The disulfide bond-containing cell sheet engineering system is expected to bring much higher cell adhesion and cell activity fulfilling the needs for clinical applications. We also look forward to the system could be applied into clinical practice in near future.

RESULTS AND DISCUSSION

The transparent polyvinylidene fluoride (PVDF) membrane were sequentially reacted with disulfide bond-containing amino acid and biopolymers after carbon dioxide plasma treatment via two stage coupling reaction. In PVDF membrane reacted with poly gamma-glutamic acid (γ -PGA), the water contact angle fell from the original untreated PVDF membrane, $70.9^\circ \pm 1.45^\circ$ to $26.1^\circ \pm 2.39^\circ$ after surface modification with γ -PGA. The water contact angle was $37.8^\circ \pm 4.87^\circ$ when cysteine was used as a reductant to cleave the disulfide bond and release the residual γ -PGA. The similar results were also found in the PVDF membrane reacted with hyaluronic acid (HA). The staining results of methylene blue, which binds to the amine group of γ -PGA, shows that the two-stage reaction is uniform with good integrity. In PVDF reacted with γ -PGA, the ESCA analysis show the element changes at every response as expected as shown in figure 1, respectively. The PVDF membrane reacted successfully with HA were also confirmed ESCA analysis. This further confirms that these membranes surface were successfully modified and by adding reducing agent, the disulfide bonds can be easily cleaved.

Cytotoxicity tests show good biocompatibility of the matrix PVDF membranes. For corneal epithelial cell sheet, the New Zealand rabbit corneal epithelium were removed. After tissue separation, multi-layer promoting agent were added to form

a multi-layered corneal epithelial cell sheet under a serum free culture condition for 10 days' cultivation, and appropriate amount of reductant were added. The multi-layered cell sheet were detached spontaneously and were transferred onto electrospun PLLA carrier to smoothly fabricate a corneal epithelial cell sheet for operation-ready. After that, almost all cells on multi-layered cell sheet was keep at survival state were confirmed by the vital assay. Further, multi-layered cell sheet were cryosectioned and then were H&E stain and that layers of cell sheet up to 5 layers were found. Both of the cornea or oral mucosal epithelial cell sheets has demonstrate some of the corneal epithelium specific k3/k12 expression also were found by immunohistochemical staining results. These results are shown in figure 2. Further, human periodontal ligament stem cell, human dental pulp stem cell and rabbit/mice endometrial stromal cell were separated, cultivated, easily detached and smoothly transferred onto carriers.

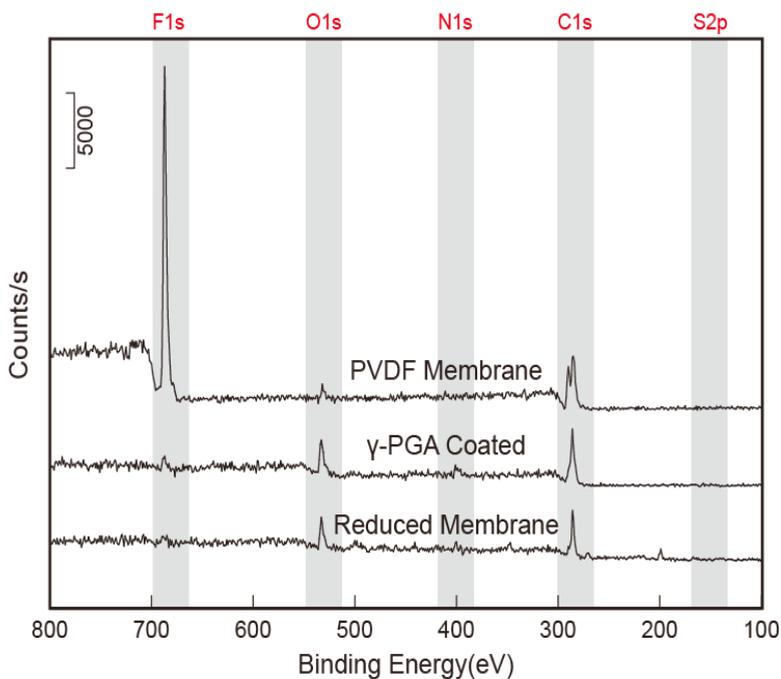


Figure 1: ESCA wide-scan spectra of pre-/post-modified

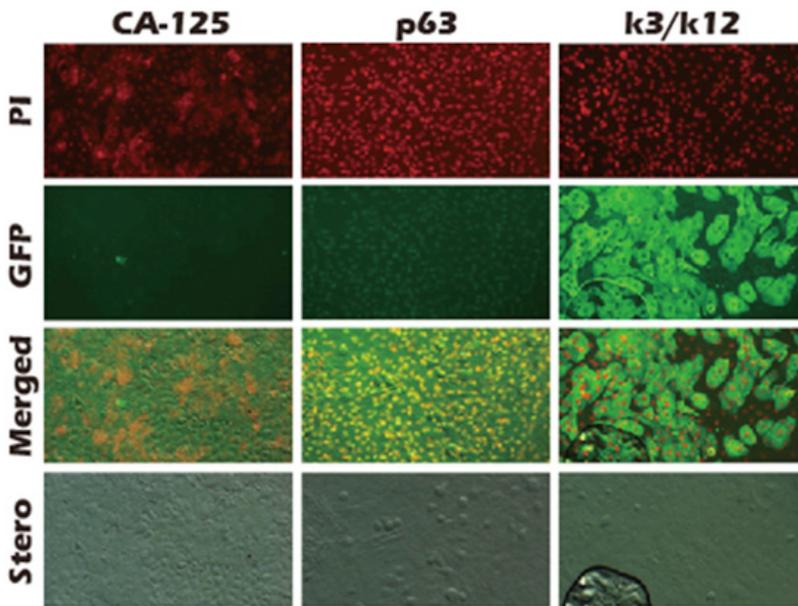


Figure 2: Immunocytochemical staining of rabbit cornea and reduced PVDF membranes. epithelial cell.

For autologous/allogeneic corneal epithelium reconstruction, the New Zealand rabbit oral mucosa epithelium were used as cell source. After tissue separation, a multi-layered oral mucosa epithelial cell sheet were cultivated under autologous/allogeneic serum culture medium after air-lifting for a week culture. After amino acidic reductant adding, the multi-layered cell sheet were detached spontaneously and were transferred onto electrospun PLLA membrane made carrier. After that, almost all cells on multi-layered cell sheet was keep at survival state were confirmed by the vital assay. For preliminary animal study, the prepared autologous oral mucosa epithelial cell sheet was transplanted into autologous rabbit right eye. That the corneal epithelium were almost recovered on transplanted right eye were compared with control site of left eye were confirmed by corneal epithelial fluorescein staining.

CONCLUSIONS AND/OR OUTLOOK

In summary, carbon dioxide plasma treatment following adding Cystine, EDC, and γ -PGA (or hyaluronic acid) generates well formed two-stage surface-modified PVDF membranes. PVDF membranes seem to be a novel and potential material in engineering

cell sheet and could be useful in harvesting cell sheets. The PVDF membranes surface were successfully modified and by adding reducing agent, the disulfide bonds can be easily cleaved were confirmed by methylene blue, water contact angle measurement and ESCA. further, we utilize our cell sheet engineering system successfully cultivated rabbit cornea epithelial cell, oral mucosal epithelial cell, periodontal ligament stem cell, dental pulp stem cell and endometrial stromal cell. It's were easily detached and be smoothly transferred onto an electrospun PLLA membrane made carrier. The preliminarily animal test results for corneal epithelium reconstruction also shows the feasibility of our system. We also look forward to the system could be applied into clinical practice in the near future.

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METALLOGRAPHIC PREPARATION AND EXAMINATION OF MEDICAL DEVICES

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Keywords: metallography, medical implant devices, specimen preparation, etching

Medical technology has developed many new devices that can be implanted into humans (*in-vivo*) to repair, assist or take the place of diseased or defective bones, arteries and even organs. The materials used for these devices have evolved steadily over the past fifty years with titanium and cobalt-based alloys replacing stainless steels. Metallographic examination has become an indispensable tool in the testing, quality control, failure studies and post-mortem analyses of these devices. This paper presents metallographic preparation techniques and results for examination of titanium-based acetabular cups and Co-Cr-Mo femoral hip stems and knees. These implants have porous metallic coatings on one side to enhance bone/metal interface adhesion by in-growth of bone into the porous coatings. The presentation also describes the preparation of nitinol (and other alloys used for stents) and illustrates its microstructure before and after going through the shape memory effect.

Metallographic work is usually conducted on new implant materials and devices to guarantee their quality or determine if the manufacturing approach planned is satisfactory; or, it is conducted on implants removed from animals to evaluate suitability or bone in-growth development. Each of these problems requires the metallographer to develop a good plan for sampling of the implant to learn what is required. Obtaining the specimens for analysis requires utilization of optimal sectioning procedures to minimize damage to the implant material. As surfaces are a prime subject for examination, encapsulation is required to promote edge retention. This can be complicated if a foam or a medicinal coating has been applied to the surface. The void space within the foam must be infiltrated with a low-viscosity epoxy to preserve the pore geometry and protect the foam during preparation. Medical coatings on implants have vastly different properties than

metals or ceramics and may not be inert to water used in the preparation sequence. Once the specimens have been encapsulated and the desired surfaces are ready to be prepared for examination, the metallographer may be faced with a relatively straightforward preparation sequence if the implant is simple in construction, that is, has no formidable surface coatings to deal with. However, in the case of implants with bonded foam surfaces or medicinal coatings, then the preparation sequence will be more challenging. Foams are often made from tantalum, a difficult refractory metal to prepare. Revealing both the substrate, which may be made from a variety of highly corrosion-resistant metals and alloys, and the Ta foam, will test the mettle of all metallographers. Medicinal coatings on metallic substrates may be water soluble, and dealing with non-aqueous abrasives, extenders, and cleaning solutions is always a chore.

As these metals and alloys tend to be highly corrosion resistant, etching them properly so that the microstructure is fully and clearly revealed may be quite difficult. In general, there are well-known, highly successful etchants for austenitic stainless steels. However, etching the Ni-free high Mn content austenitic stainless steels is very difficult, all the more so when they have been cold worked in manufacture. Co-based alloys are very popular for implants and, while etchants for them exist, it is still a challenge to fully reveal their structure with clarity. Grain size rating of any twinned FCC metal is always difficult as many etchants reveal only a portion of the grain and twin boundaries. Co-based alloys are FCC, although pure Co is hcp, and as they are highly corrosion resistant, etching them properly is difficult. Commercial purity (CP) Ti can be examined as-polished using polarized light – but only if all the preparation-induced damage is removed. Preparation philosophy must be to prepare the specimens totally free of any preparation-induced damage; “just good enough” preparation is not a viable concept for this work.

MULTIFUNCTIONAL NANOMATERIALS FOR SMART RELEASE

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Keywords: mesoporous bioglass, therapeutic ions, tissue regeneration, drug release

INTRODUCTION

The use of organized mesoporous materials in biomedical applications continues to gain interest due to their well-defined nanoporous structures offering them the ability to store and release different molecules based on their nanopore size and shape. They offer several advantages such as large incorporation of drugs, control on the drug release kinetics, excellent payload stability. In addition, mesoporous inorganic materials are amenable to surface functionalization, which allows tuning of the hydrophilicity/hydrophobicity character, encapsulation of a variety of drugs, combination with other vehicles (e.g. polymer), but can also impart stimuli-responsive and/or targeting properties.

Mesoporous bioactive glasses (MBGs), which combine the textural parameters of ordered mesoporous matrices with the properties of conventional bioactive sol-gel glasses, have received increasing attention as bone-tissue regeneration systems [1]. The ambition is to impart other biological functions, including anti-bacterial activity, as well as stimulation of osteogenesis and angiogenesis, by incorporating therapeutic metallic elements and drugs [2].

This talk will also introduce the H2020 project MOZART, whose general objective is

to develop a library of mesoporous glasses doped with selected ions, to be used as a new, smart platform technology for future, highly targeted therapies in pathological bone and skin.

With the aim to develop a mechanism of controlled, triggered release of the payload based on pH changes, the drug-loaded nanomatrices have been coated with self-immolative polymers (SIPs) that close the gates of the pores, avoiding the premature release of the payload, and disassemble upon local pH changes associated with pathological states.

RESULTS AND DISCUSSION

MBGs based on the SiO₂-CaO system and containing different doping ions (Cu²⁺, Sr²⁺, Ce³⁺) have been synthesized both by an ultra-sound assisted sol-gel method and by an aerosol-based spray-drying process in order to obtain different particle size and morphology. FESEM image of spray-dried Cu-doped SiO₂-CaO (Cu-MBG_SD) mesoporous glasses (Figure 1, A) shows micro-sized spherical particles, with size mostly ranging between 500 nm and 5 μm, slightly aggregated. The particles prepared by ultra-sonication (Cu-MBG_US) showed spheroidal shape and size of about 200 nm, without large aggregates (Figure 1, B). The EDS quantitative analysis on both samples confirmed that their compositions are very close to the theoretical one. TEM observations of sample prepared by ultra-sonication showed that the nanoparticles contain mesopores throughout their inner structure, in the form of a worm-like system (Figure 1, C). Quantitative analysis using EDS mapping was used to explore the element distribution in the sample and showed that Cu ions, like the other elements, were evenly distributed within the whole of the particle (Figure 1, D).

High specific surface areas, about 300 m²/g for spray-dried MBGs and about 600 m²/g for MBGs prepared by ultrasonication-assisted sol-gel method, and homogeneous pore size distribution were obtained by N₂ adsorption analyses.

Excellent bioactivity, in terms of apatite-like forming ability in SBF, was found for Cu-containing samples, showing that the incorporation of metal does not affect the *in vitro* bioactive response. A sustained and almost quantitative release of Cu²⁺ ion, measured by inductively coupled plasma-atomic emission spectrometry (ICP- AES), was observed for Cu-doped MBG samples, with released concentrations, which on the basis of the literature [3], are suitable for inducing osteogenesis.

The results of antibacterial tests (Figure 2) showed that the incorporation of Cu²⁺ ions into the bioglass structure induces a strong antibacterial effect, a fundamental

function for preventing implant-related infections, avoiding the need for systemically administered antibiotics.

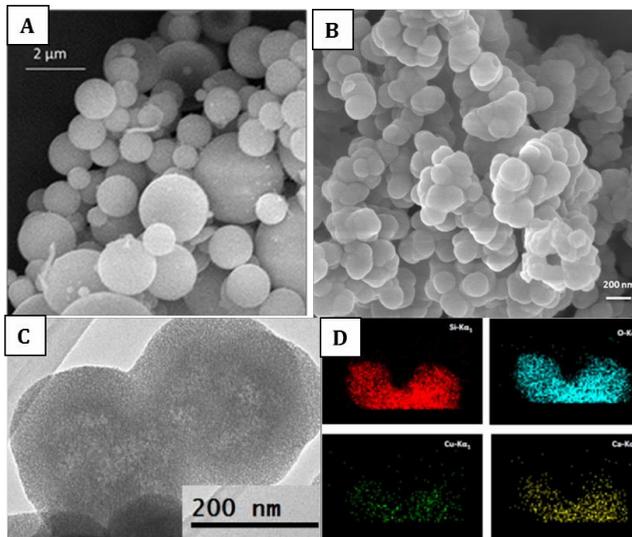


Figure 1: FESEM micrographs of Cu-doped MBGs prepared by aerosol-assisted (A) and ultrasonication sol-gel synthesis (B). TEM image (C) and EDS analysis mapping (D) of Cu-MBG_US.

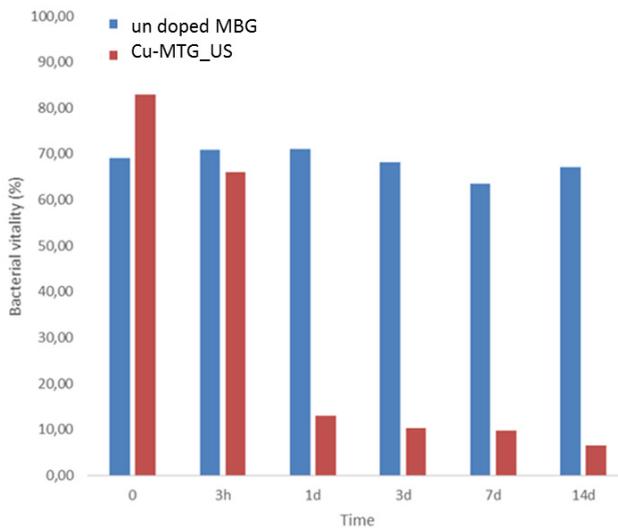


Figure 2: Antibacterial effect of Cu-MBG_US against *Escherichia coli* strain.

CONCLUSIONS

Novel MBG matrices doped with controlled amount of therapeutic ions, characterized by high surface area, fully accessible nanoporosities, excellent bioactivity and antimicrobial properties, were prepared by different synthesis procedures. These nanomaterials will find various applications as multifunctional therapeutic agents for tissue regeneration.

ACKNOWLEDGEMENT

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THE NEXT GENERATION OF IMPLANTS: USING NANOMEDICINE WITHOUT DRUGS TO CONTROL CELL RESPONSES

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Keywords: Nanomedicine, nanotechnology, tissue engineering, bacteria, infection, inflammation

INTRODUCTION

Nanomedicine (or the use of nanotechnology in medicine) has begun to revolutionize medicine with several FDA approved products for implantation. This is because the fundamental approach to using nanotechnology in medicine is to alter only the size of material and/or surface features without changing implant chemistry. This provides an easy way for FDA approval since new chemistries are not produced, just alterations in their size. One of just many examples of this approach is present here, dealing with skin regeneration. One of the most predominant symptoms of skin aging is wrinkle formation, which results from both intrinsic aging and environmental damage, such as chronic exposure to UV radiation. Current anti-aging and anti-wrinkle materials often induce a toxic response, which results in inflammation to increase tissue growth under the skin. Although effective, a much safer and more effective way to alleviate the effects of aging and thereby wrinkle formation needs to be developed. Several biomimetic peptide sequences¹ (KTTKS, GQPR, GHK, etc.) have been identified as structural mimics of collagen type I to be effective in stimulating the synthesis of key constituents of the extracellular matrix by fibroblasts, however, they typically suffer from poor skin penetration due to the existence of the stratum corneum. Incorporation of cell penetrating peptides (CPPs) has been an emerging strategy to transport a variety of cargo across cellular membranes in a dose dependent manner.¹ In this study, peptide amphiphiles that consist of both CPPs and biomimetic sequences were designed, synthesized and characterized. The abilities of these peptide

amphiphiles to penetrate the skin, to reduce inflammation, and to promote fibroblast functions (specifically, increasing the adhesion, proliferation, collagen synthesis, and decreasing elastase and collagenase synthesis) were determined.

METHODS

Material characterization: Self-assembled peptide amphiphilic nanoparticles (APNP) were characterized by zeta potential to determine their charge, by dynamic light scattering to examine their size, and by TEM to observe their morphology.

Cell viability assay: To determine cell viability, human dermal fibroblasts were seeded at a density of 10,000 cells/cm² in 96-well plates and were subsequently co-cultured with peptide amphiphiles at various concentrations (12/20/40/80/160 μM) for 24 hours. MTS assays were used to determine cell density after incubation. Briefly, 20 μl of an MTS dye solution was added per 100 μl of solution, and the absorbance readings were taken at 490 nm after 4 hours of incubation. Cell density was then determined with correlation to a standard curve.

Cell adhesion and proliferation: To determine cellular adhesion/proliferation, keratinocytes (ATCC® PCS-200-011™) and human dermal fibroblasts (Lonza, CC-2511) were seeded at a density of 10,000 cells/cm² and cultured respectively in keratinocyte medium supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (P/S, Hyclone), and DMEM supplemented with 10% FBS and 1% P/S. Both types of cells were incubated under standard cell culture conditions (37°C, humidified, 5% CO₂/95% air) for a certain period of time (4h for adhesion; 1, 3, and 5 days for proliferation). The MTS assays were used to determine cell density after incubation.

Bacteria inhibition: Bacteria (*S. aureus*, MRSA, and *S. epidermidis*) were seeded in a 96-well plate at a density of 10⁵ CFU/ml, and were then combined with varying APNP concentrations to obtain a final concentration 0, 12, 20, 40 and 80 μM. The cells were then allowed to incubate at 37 °C for 24 hours. The final density of bacteria was finally determined by colony counting.

Bacteria growth curves: Bacteria were seeded in a 96-well plate at a density of 10⁶ CFU/ml, and were then combined with varying APNP concentrations to obtain a final concentration 0, 2, 4, 8, 12, 20, 40 and 80 μM. The well plate was then allowed to incubate at 37 °C inside a spectrophotometer under static conditions. OD600 measurements were taken every 2 minutes for 24 hours to establish the speed of proliferation and shape of the bacterial growth curve.

Skin penetration/permeation: Skin penetration and permeation were determined using the Static Franz Diffusion Cell (FD-C) with porcine skin as the membrane between donor and acceptor

compartment due to their resemblance to human skin. Peptide amphiphiles were applied in the donor compartment, and PBS was used as the receptor solution. The donor compartment was sealed with aluminum foil and the system was maintained at 37°C in a water bath. The skin samples were fixed and sectioned to examine penetration using confocal microscopy. Measurement of total collagen: The sircol soluble collagen assay (Biocolor, UK) was used to quantify the total soluble collagen after 7, 14 and 21 days of culturing. All experiments were conducted in triplicate and repeated at least three different times. Statistical differences between means were determined using ANOVA followed by a student t-test where $p < 0.01$ was considered statistically significant.

RESULTS AND DISCUSSION

The LC50 value of APNPs for human dermal fibroblasts was found to be around 76 μM , and no toxicity was identified for the same cells at a concentration of up to 12 μM (Figure 1). Also, it was found that these peptide amphiphiles as low as 2 μM in concentration have the ability to both delay the time it takes for bacteria to reach exponential growth phase and decrease bacteria density after one-day of culturing for gram positive bacteria (both normal and antibiotic-resistant strains, Figure 2). The effects increased in a dose dependent manner. Last, but not least, it was found out that APNPs were able to promote fibroblast cell proliferation as well as total collagen synthesis over the long term. In this manner, this study represents an effort to not alter chemistry but change the fundamental size of a material which shows maintenance of tissue forming cell functions and decreased bacteria functions without using drugs; such an approach will accelerate FDA approval for implantation.

CONCLUSIONS

Through the above experiments, various peptide amphiphiles (with a chemistry already approved by the FDA for implantation) were identified that can be added to current skin formulations to promote the penetration of active ingredients, to increase fibroblast growth and decrease bacteria growth, criteria necessary for improved anti-aging and antimicrobial agents. However, in contrast to traditional peptide amphiphiles, the study here modified dimensions into the nanometer regime and showed decreased bacteria growth while maintaining mammalian cell functions. Such an approach will accelerate approval from the FDA for implantation.

ACKNOWLEDGEMENTS:

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ORAL

CERAMICS: AN OFFER THE BODY CAN'T REJECT

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Keywords: Ceramic biomaterials, oxides, nitrides, calcium phosphates, carbon.

The term “ceramic biomaterials” identify a broad class of materials, including some glasses and calcium phosphates, oxides, nitrides and carbon-based materials. From the point of view of their in-vivo behavior these ceramics can be classified as bioresorbable, bioactive or (nearly) inert, and a number of devices were developed to exploit such characteristics at their best.

The start of the development of ceramic biomaterials can be set in the second half of 1800. In 1852 Antonius Mathijssen published the results achieved using bandages soaked in plaster of Paris (calcium sulphate) to treat fractured bones. The results obtained by Mathijssen suggested to Dreesmann, working in the Trendlenburg clinic in Bonn, Germany to develop a blend of calcium sulphate and phenol to fill bone defects and stimulate bone remodelling. Calcium sulphate is still in clinical use as resorbable material in the treatment of bone defects. In the same years Themistocles Gluck performed the first arthroplasties using ivory, a natural ceramic still in use on the 1960s in some far Eastern Countries for the construction of hip replacements.

The successive advancement took place about 1920, when Albee and Morrison demonstrated the faster bone remodelling in rabbits after injection of solution of β -TCP. The quest for a load bearing bone substitute oriented the attention of some reserchers towards ceramic oxides, especially alumina and zirconia. The first patent on alumina as a biomaterial was issued on 1933 in Germany by Rock but it was not followed by practical applications. It was only during the second half of the 1950s that Lyman W. Smith started the development of a load-bearing bone substitute for segmental substitution of long bones. On 1963 he patented Cerosium, a 65% porous alumino-silicate filled with epoxy resin. In the same years, Eyring and Campbell were testing alumina spinal spacers.

The outcomes of these device were usatisfactory, mainly for the high content

of calcium and sodium impurities in these early alumina. The introduction of the high-alumina precursor Al₂O₃ by Degussa in the first half of the 1960s gave a new momentum to the clinical use of alumina: in 1965 Sami Sandhaus patented the dental implant CBS (Crystalline Bone Screw) an alumina fixture that is the first load-bearing bioceramic device used in clinics. In the same years Paul Boutin was carrying out studies that led him to introduce in hip arthroplasty his alumina ceramic-on-ceramic bearing in 1970.

The promising results obtained by Sandhaus and by Boutin fostered the research on ceramic biomaterials, carried out especially in the USA, Germany and Japan. In the USA Hulbert, Klawitter and Driskell, developed alumina dental implants in the framework of a program granted by the Department of Defense. The main result was the Synthodont dental system, in clinical use from 1975. In Germany the Ministry of Research managed a comprehensive research program on bioceramics involving the main manufacturers of the Country (Friedrichsfeld, Rosenthal, Feldmühle). This resulted in the development of several models of dental implants, including the so-called Tübingen-Type that had a positive clinical success. In addition, the German researchers developed a number of devices made out alumina like e.g. keratoprotheses, parts for the middle-ear ossicular chain, implantable injection ports. However, the most relevant result obtained by the German researchers was the development of the hip replacement bearings made out BIOLOX alumina, a ceramic biomaterial in clinical use since 1974 that underwent many improvements since then. Its fourth-generation (BIOLOX *delta*) is the most widely used ceramic biomaterial worldwide so far.

In the same time, Japanese researchers were developing devices based on ceramic biomaterials using a peculiar approach. To overcome the mechanical limitations of 1970' polycrystalline alumina, they focussed their activity on single-crystal alumina (synthetic sapphire). Using such materials they developed a number of different dental implants, that were manufactured by Kyocera Corp. under the trademark BioCeram and used in clinics in Japan and in the USA. Japanese researchers, and especially Kawahara and Oonishi, are distinguished especially for the huge amount of work performed in the development of ceramic biomaterials. Single-crystal alumina was used also for the stem of all-ceramic hip replacements, and in many other orthopedic devices. In addition, Japanese researchers devoted a significant effort to the development of ceramic knee replacements, a device that in Italy and Germany has been object of relevant developments during the last years and is now entering in clinical use in the EU.

In the same years Bokros was proposing the use of carbon (LTI-Si carbon) in

dental implants and heart valve prostheses, as well as coating of the surface of hip endoprostheses. Diamond-like carbon (DLC) has been extensively tested as wear resistant coating for orthopedic devices, without successful clinical applications so far. On the other hand, turbostratic carbon is employed since a long with very good clinical outcomes in heart valves for its haemocompatibility and antithrombogenic behavior. Carbon fiber reinforced polymers (CFRP) have been investigated for use in joint replacements, but this application appears to have been abandoned so far.

The development around 1970s of Plasma Spray (PS) technology paved the way to the use of ceramic coatings. Early applications were addressed to the protection of metallic implants from corrosion, then the attention shifted to the application of hydroxyapatite and other calcium phosphate as osteoconductive coatings thanks to the results achieved by distinguished scientist like among others Osborne, Kokubo, Le Geros, De Groot, Hugabaert, Bonel, Daculsi. The introduction of Vacuum PS (VPS) as the set up of dedicated ceramic feedstock during the mid 1990s gave a strong contribution to the production of the reliable osteoconductive calcium phosphate coatings in clinical use today. In addition, calcium phosphates with different formulations have a wide field of applications in medicine as bone substitutes and as scaffolds for guided tissue regeneration.

During the 1990s zirconia was introduced in the biomaterials scene. It was used mainly in orthopedics until the year 2000 when its use was practically abandoned. Zirconia (Y-TZP) is now used in dentistry, for the production of dental implants, and for the structure of crowns, bridges and of full arch dentures. Zirconia is used in orthopedics in ceramic composites with alumina, performing as the toughening. These toughened composites (ZTAs either ATZs, depending on their matrix) are the state-of-the-art of ceramics for joint replacement bearings. The outstanding mechanical behavior of these composites are making feasible the production of new ceramic devices to replace knee, ankle, shoulder joints, as well as applications in other anatomic districts. Also the traditional application of ceramic biomaterials in hip replacement bearings had a new momentum thanks to the introduction of ceramic composites in the clinical practice.

An overview on ceramic biomaterial must not forget the use of nitrides of titanium as coating in joint replacement bearings to limit their wear. Also silicon nitride, that has been investigated for use as a biomaterial since the late 1970s has been proposed for use in joint replacement bearings. Clinical trials now in progress in the USA will assess the validity of the material in this application.

SUPER FIRMUM FUNDAMENTUM: THE APPROACHING RENAISSANCE FOR CALCIUM PHOSPHATES IN BIOMEDICINE

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Calcium phosphate was selected by Nature to be the firm foundations of our bodies. This has served as an invaluable inspiration for materials scientists who have attempted to discover in this material more potential than meets the eye. Efforts are currently being made to expand the application repertoire of calcium phosphates beyond their use as traditional bone fillers or tissue engineering construct components that impart osteoconductivity and high compressive strength. The application of calcium phosphates for sustained drug delivery, gene and anticancer therapies, antibiofilm coatings and hard tissue regeneration has been intensely explored recently. All this plethora of applications for calcium phosphates that are now in the R&D stage are the consequence of the immense structural complexity of this material, which is being directly reflected in its ability to display an array of exciting properties under precise synthesis regimens. Like water, *la principessa* of peculiarities in the realm of liquids, calcium phosphate deserves the epithet of *il principe* of peculiarities in the realm of solids. Its protean nature and the applicative potentials arising from this peculiar nature will be described in this lecture. Foreseen on the horizon will be a new generation of materials for therapeutic and regenerative applications, containing only precisely designed calcium phosphates and substituting for the role of expensive bone growth factors, antibiotics, viral vectors and polymers.

DOPED CALCIUM PHOSPHATE BONE CEMENTS AND REAL-TIME MONITORING OF THEIR HARDENING MECHANISM

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Keywords: calcium phosphates, cements, doping ions, bone, materials for orthopaedics and dentistry, functionalized bone graft materials

INTRODUCTION

Nowadays, considerable efforts have been focused on various calcium phosphate based cement compositions (CPC), developed to restore the damaged human hard tissues, like vertebrae and osteoporotic bone. Furthermore, CPC materials are widely used as synthetic substitutes for non-load bearing fractures, bone defects and teeth reconstruction, due to their chemical similarity to the mineral component of the natural hard tissue. However, the proposed up to now cement formulations can be improved by endowing cements with additional properties, requested for a certain application field. For example, the bone graft associated infections can be prevented by doping the synthetic materials with Ag⁺ ions at low (non cytotoxic) concentrations. This way seems to be more appropriate, because the antimicrobial agents could be provided directly at the implantation site. Another interesting functionalization option are Zn²⁺ ions - an essential trace element participating in a variety of cellular processes, including DNA synthesis, behavioural responses, bone formation and growth and wound healing. Moreover, zinc plays an important role in gene expression and in the regulation of cellular growth and differentiation. To this end, Zn²⁺ is known to possess antibacterial, osteo- and angiogenetic properties, enhancing the proliferation of osteoblastic cells.

The aim of this research is to develop functionalized cements for several bone grafting applications, among them those mentioned above.

RESULTS AND DISCUSSION

A cement system is generally composed of one or several powder components and a hardening liquid. After their mixing, the interaction takes place, followed by cement's crystallization and hardening. In our previous studies, we demonstrated that CPC hardening mechanism is much more complex than expected, characterized by several processes, such as chemical reactions, phase transformations (new products and intermediate phases) and amorphous-into-crystalline conversion (i.e. the primary and secondary crystallization process). In order to monitor in real time all these processes, in our lab, the Energy Dispersive X-Ray Diffraction (EDXRD) technique (50 KeV X-Ray source) is applied, being very suitable for this task [1-6]. In Figure 1, a typical 3D diffraction map, allowing to follow *in situ* structural changes occurring upon evolving cement systems and the kinetics of their transformation/hardening process, is shown. For comparison, the results obtained at a high brilliance Synchrotron Radiation Facility, having the advantage of higher energy source (up to 90 KeV) and allowing to perform experiments with much higher spatial and temporal resolution, are presented. As can be seen from Figure 1, our laboratory time-resolved EDXRD measurements, even if compared to the Synchrotron facility, still can elucidate hardening process with a good level of accuracy and allow to perform long-term *in situ* investigations.

In the present-day clinics, the injectability of CPCs is of crucial importance in vertebroplasty and kyphoplasty for the delivery of cement. The injectability of cements strongly depends on the post-mixing time interval relative to the cement setting reaction (Figure 2). The setting process could be monitored in real time by the laboratory EDXRD.

In Figures 3 and 4, several examples are given, i.e. the 3D diffraction maps of OCP (octacalcium phosphate), DCPD (dicalcium phosphate dehydrate) and chitosan based cements, supported by the SEM images, are shown. The OCP cements were chosen, since OCP is assumed to be a precursor of the final substituted HA phase and of the biomineralisation process *in vivo*. It should be stressed that in this work, a special attention was paid to the *in vitro* behaviour of the nanograin size cements, being of great interest for biomedical applications due to their biocompatibility, osteoconductive and possibly osteoinductive properties.

In addition, several TCP-Ag (tricalcium phosphate) cement systems (with different Ag wt%) were investigated by the EDXRD, complemented by SEM, FTIR, and AES (Atomic Emission Spectroscopy) data. Antibacterial tests proved the inhibitory

effect towards pathogenic *Escherichia coli* of some TCP-Ag cement formulations. The obtained results are summarized in Figure 5.

In Figure 6, the Zn^{2+} (0.6 wt%) doped resorbable β -TCP cement hardening process, followed by EDXRD, is shown.

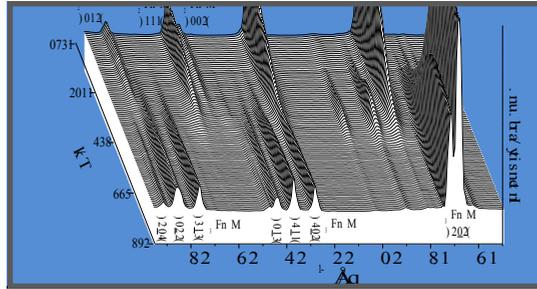


Figure 1: EDXRD experimental setup and its possibilities versus Synchrotron Radiation facility.

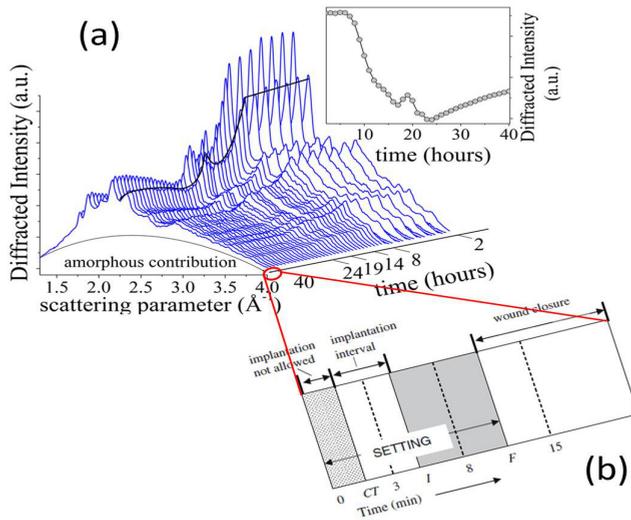


Figure 2: (a) Sequence of diffraction patterns collected during the hardening of the DCPA (dicalcium phosphate dehydrate amorphous) cement. (b) Diagram of the setting parameters relevant for a CPC: CT- cohesion time; I- initial setting time; F- final setting time.

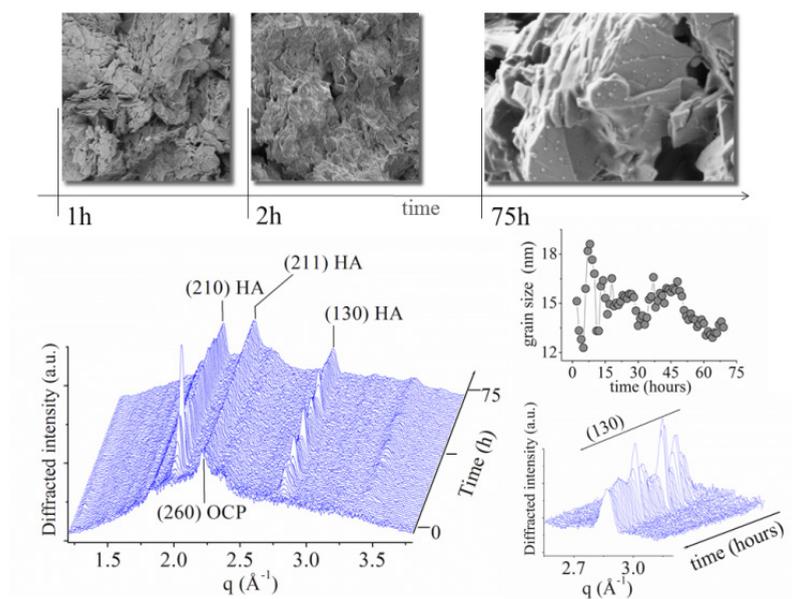


Figure 3: Sequence of EDXRD patterns collected upon the OCP – chitosan cement [4]. SEM images (75x) were taken at certain time intervals.

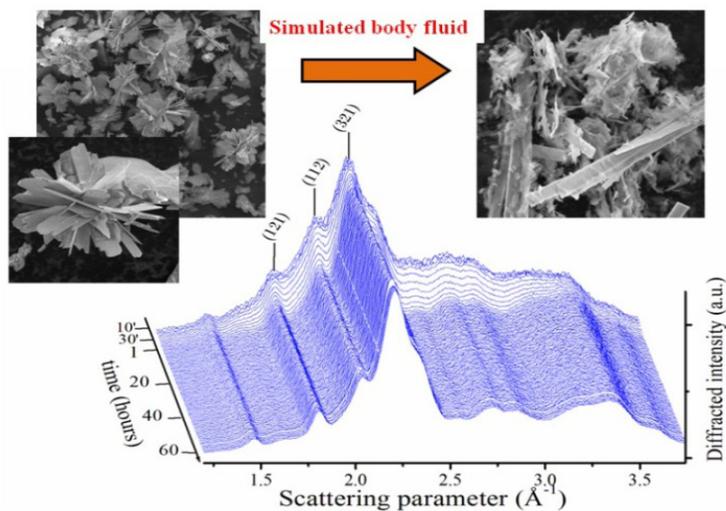


Figure 4: Sequence of EDXRD patterns collected upon the OCP – DCPD cement. SEM images (5x) correspond to certain time intervals [2].

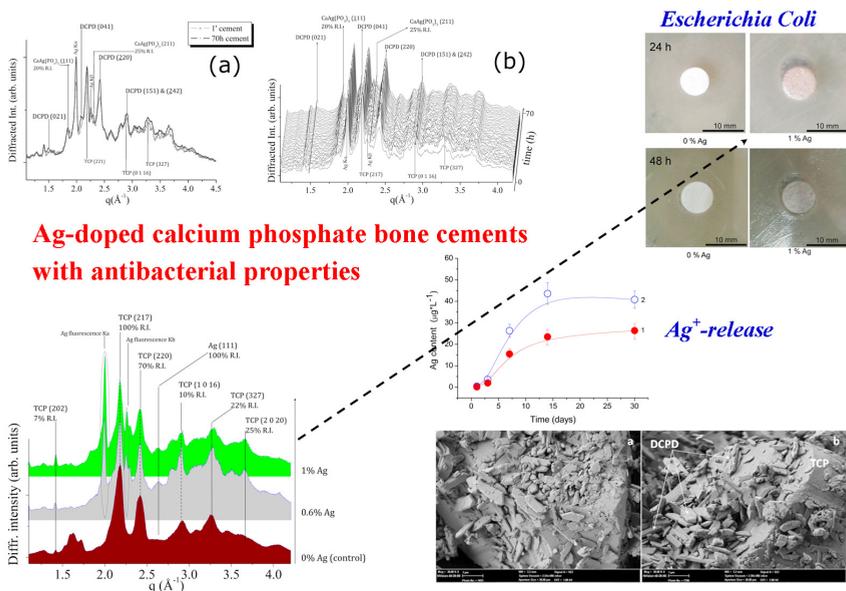


Figure 5: Recent EDXRD results obtained for Ag⁺ doped resorbable β-TCP cements [7].

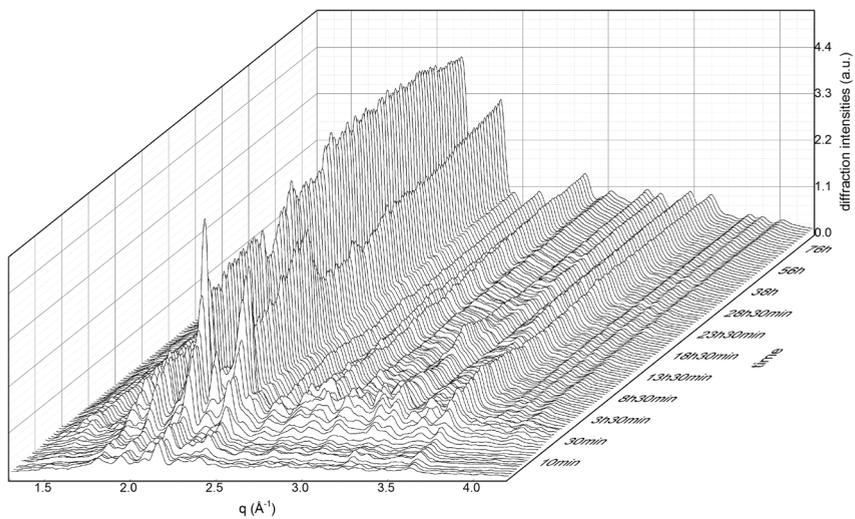


Figure 6: 3D map of Zn²⁺ doped resorbable -TCP cement [8].

Materials science's concept «composition-structure-property», in our case, can be detailed as chemical and phase composition of the initial and final CPC systems – micro- and nano-structure of material (grain size)- and its various functional properties, one of them being the osteogenesis promotion.

CONCLUSIONS AND/OR OUTLOOK

The *in situ* time-resolved EDXRD monitoring of the CPC hardening process is expected to deepen the knowledge of their setting process and hardening mechanism, bringing outcomes in the field of application of these materials, like bone tissue engineering and regeneration. This will hopefully lead to a significant improvement of the CPC's characteristics required for modern orthopaedic and dental implant surgery for the replacement and reconstruction of the damaged human bone tissue, decreasing the rehabilitation time and increasing the life quality of post-operation patients.

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OSTEOCONDUCTIVE CERAMICS BASED ON $\text{Ca}_{3-x}\text{Na}_{2x(1-y)}\text{K}_{2xy}(\text{PO}_4)_2$: FABRICATION AND RESORBABILITY TESTING

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Keywords: bioceramics, double calcium and alkaline phosphates, rhenanite, solubility, 3D-printing

INTRODUCTION

Biomaterials based on hydroxyapatite (HAp, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) or/and more soluble tricalcium phosphate (TCP, $\text{Ca}_3(\text{PO}_4)_2$); are widely used for bone tissue engineering, but still does not meet all the clinical requirements (bioresorption, strength, fracture toughness, etc.). We expected to elaborate double/ternary phosphates of calcium and alkali metals (sodium and potassium) for ceramic bone scaffolds production with enhanced level of solubility matching that of materials based on HAp or/and TCP.

3D-printing is a promising technique to fabricate bone scaffolds of complex shape. Ceramic scaffolds with Gyroid structure and high porosity obtained via stereolithography can enhance osteoconductive properties.

Results and Discussion

Ceramics with pre-determined pore size (50 μm and more) and overall porosity >80% with Gyroid architecture were fabricated via stereolithography. Gyroid structure is used to enhance osteoconductive properties of the ceramic scaffolds due to its high permeability.

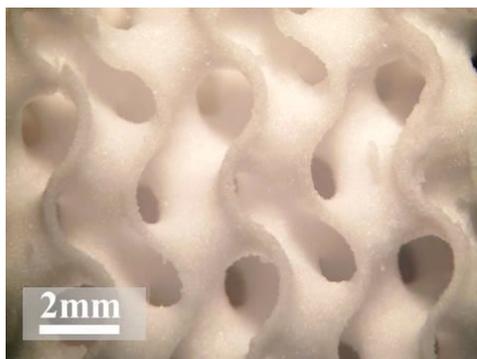


Figure 1. 3D-ceramic with Gyroid structure based on double phosphates of calcium and alkali metals

Kinetics of dissolution (resorption) of crashed 3D-printed ceramics (down to 100 μ m) was studied in several media at definite pH level with the help of automatic titrator Mettler Toledo T50 in pH-stating mode. Thermodynamic modelling of the ionic equilibria in solutions containing Ca^{2+} , PO_4^{3-} and citric anion cit^{3-} at different pH was done to make a right choice of the pH values for the dissolution tests. At high concentration of calcium ion - $[\text{Ca}^{2+}] > 15\text{mM}$, the dissolution proceeds in incongruent manner, so the less concentrations was chosen. It was shown that at $[\text{Ca}^{2+}] = 3\text{mM}$, $[\text{PO}_4^{3-}] = 2\text{mM}$ ($\text{Ca}/\text{P} = 1.5$ as in TCP) and $[\text{cit}^{3-}] = 3\text{mM}$ one cannot avoid formation of apatite phase at $\text{pH} > 6.3$. It was shown that at pH level of 6 the kinetic of dissolution is too sluggish, but at $\text{pH} = 4$, dissolution is running too fast, so there is no chance to distinguish any difference between the samples under study. Thereby, $\text{pH} = 5$ was selected for the dissolution (resorption) tests, by and large, this level of pH is rather close to that one produced by osteoclasts within resorption lacuna during bone resorption.

Dissolution curves indicate that the ceramic sample with higher amount of high-temperature sodium rhenanite phase "A" ($\text{Ca}_{2.5}\text{Na}(\text{PO}_4)_2$) dissolves faster than other samples. Quenched samples have higher dissolution rate due to higher amount of high-temperature phase compared to the naturally cooled samples with the same composition. Dissolution rate of the "A" phase is higher than for alpha-TCP, being consistent with our simulation of solubility products for all the phases under study based on thermodynamic assessment of their lattice energies according to volume based thermodynamic approach. [1]

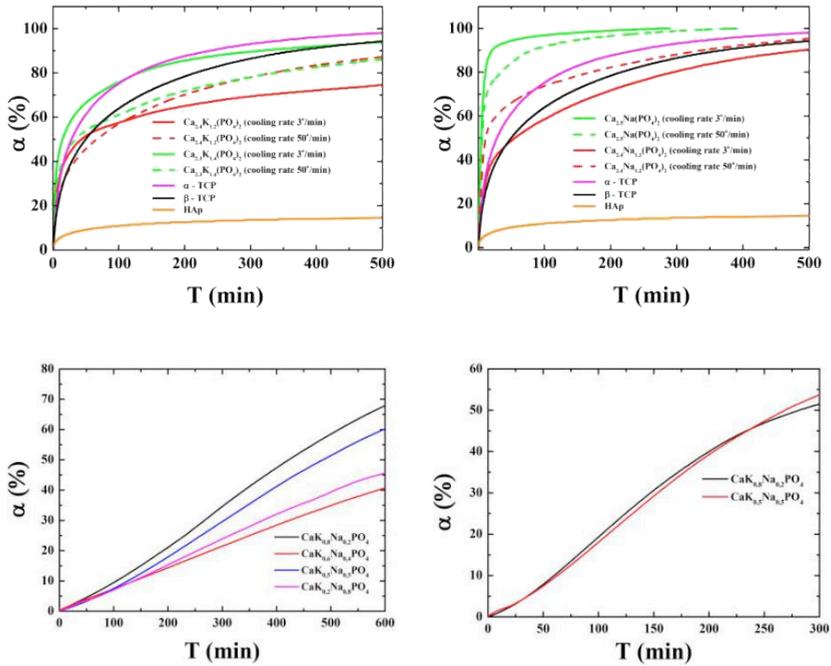


Figure 2: Kinetics of resorption a) ceramics based on HAp (orange), b-TCP(black), a-TCP (purple), $\text{Ca}_{2.5}\text{Na}(\text{PO}_4)_2$ (green), $\text{Ca}_{2.4}\text{Na}_{1.2}(\text{PO}_4)_2$ (red) (solid line – cooling rate 3°/min, dashed line – cooling rate 50°/min); b) ceramics based on HAp

(orange), b-TCP(black), a-TCP (purple), $\text{Ca}_{2.4}\text{K}_{1.2}(\text{PO}_4)_2$ (green), $\text{Ca}_{2.3}\text{K}_{1.4}(\text{PO}_4)_2$ (blue); c) ceramics based on $\text{CaK}_{1-x}\text{Na}_x\text{PO}_4$: $x=0.2$ (purple), $x=0.5$ (blue), $x=0.6$ (red), $x=0.8$ (black); d) powders of $\text{CaK}_{1-x}\text{Na}_x\text{PO}_4$: $x=0.5$ (red), $x=0.8$ (black)

Solubility of the potassium rhenanites is related to the amount of the high-temperature phase alpha- CaKPO_4 , as well. However, material based on $\text{Ca}_{2.5}\text{K}(\text{PO}_4)_2$ has a lot of the “X” phase with apatite-like structure [2] and, therefore, the lowest solubility in that system (according to the thermodynamics estimation). Increasing K^+ cation amount in the $\text{Ca}_{(3-x)}\text{K}_x(\text{PO}_4)_2$ means higher solubility of the material, because this change leads to decreasing of the “X” phase amount and increasing of high-temperature alpha- CaKPO_4 phase.

CONCLUSIONS AND/OR OUTLOOK

Osteoconductive bioceramics with Gyroid architecture based on double

phosphates of calcium and alkaline metals (potassium or sodium) were fabricated by stereolithographic 3D-printing.

The study also demonstrates variation in solubility kinetics of ceramic materials based on the double phosphates. It was shown that in the materials under study can be arranged according to the following row: HAp < TCP < X(=Ca₈K₂(PO₄)₆) ≈ TCP / CaNaPO₄ < TCP < CaKPO₄ < A(=Ca₅Na₂(PO₄)₄) / CaNaPO₄

ACKNOWLEDGEMENTS

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COVALENT ANCHORING OF VITRONECTIN SEQUENCES TO BIOCERAMIC FOAMS FOR BONE-TISSUE ENGINEERING

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Keywords: Bioceramics, bioactive peptides, selective functionalization, bone tissue-engineering.

INTRODUCTION

Bioceramic foams, typically containing Ca or Ca-Mg silicates, such as wollastonite (CaSiO₃) and diopside (CaMgSi₂O₆), can be obtained from the thermal treatment of preceramic polymers (silicone resins) containing micro- and nano-sized filler powders [1-2]. This innovative process is advantageous because of its simplicity, the limited processing temperature and the microstructural homogeneity. These foams can simulate the natural porous internal structure of human bones and consequently can be considered as promising scaffolds for bone tissue engineering [3]. Nowadays, the key role of many physiologically molecules in cell adhesion and growth has been elucidated: the acquired knowledge opens the perspective of a new approach to surface treatments, i. e. the so-called “biochemical functionalization” to improve surface-to-cells interactions. Several adhesive peptides have been individuated: in particular, a nonapeptide (HVP) from the h-Vitronectin protein (sequence 352-360) is able to enhance osteoblast adhesion through an osteoblast-specific mechanism, involving interaction between membrane glycosaminoglycans (GAGs) and the heparin binding sites on extracellular matrix (ECM). The peptide HVP has been used to design biomimetic surfaces using both unselective or selective grafting [4-8]. In this study, polymer-derived silicate foams were covalently and selectively functionalized with adhesive peptides. In addition to HVP sequence, a dimeric analogue (2HVP) has been tested in order to increase ionic interactions with cellular GAGs. On the other

side, we have synthesized two retro-inverted sequences [9] of Vitronectin peptides (DHVP and 2DHVP) in an effort to avoid the enzymatic degradation of HVP peptide, observed in serum-containing solution. Crushing strength test on functionalized scaffolds were carried out to study the influence of functionalization reactions on the mechanical properties of bioceramic foams. H-osteoblast adhesion and proliferation assays were carried out to compare the differently decorated bioactive foams.

RESULTS AND DISCUSSION

The crushing strength of foams was measured at room temperature with a cross-head speed of 1 mm/min. The results, reported in Figure 1, show no significant decrease of mechanical strength at crushing due to functionalization treatment, with average values in accordance with the literature data [2].

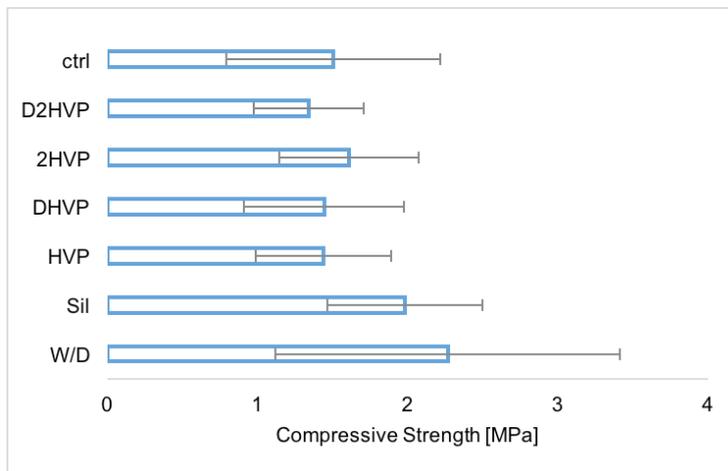


Figure 1: Crushing strength test. “Ctrl” refers to foams functionalized with a not-adhesive peptide. “Sil” refers to silanized surface, while “W/D” are not-treated Wallostonite-Diopside foams.

H-Osteoblast (3×10^5 cells/sample) were seeded onto bioceramic scaffolds (35 mg samples) and incubated at 37°C for 2 h. Cellular adhesion was assessed by MTT assay. In Figure 2a the results of adhesion assays are reported: the functionalization with the four adhesive peptides doesn't improve h-osteoblast adhesion in comparison with silanized samples or foams functionalized with a not-adhesive peptide (Ctrl). This result is in disagreement with previous ones obtained on functionalized titanium oxide or glass surfaces: probably other adhesion assays at different time points will be

carried out to confirm this experimental evidence. The results of proliferation assay at 4 days after seeding are reported in Figure 2b.

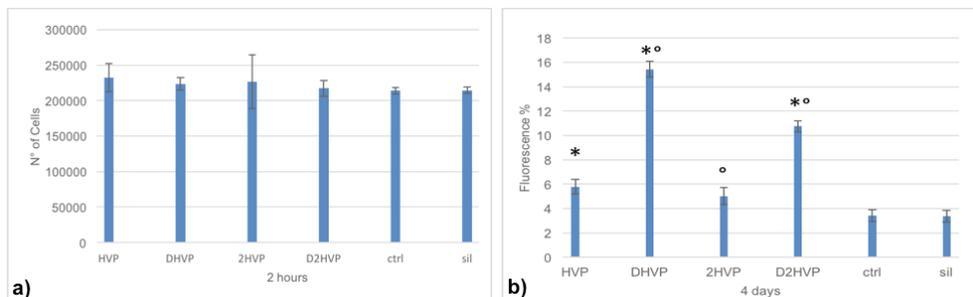
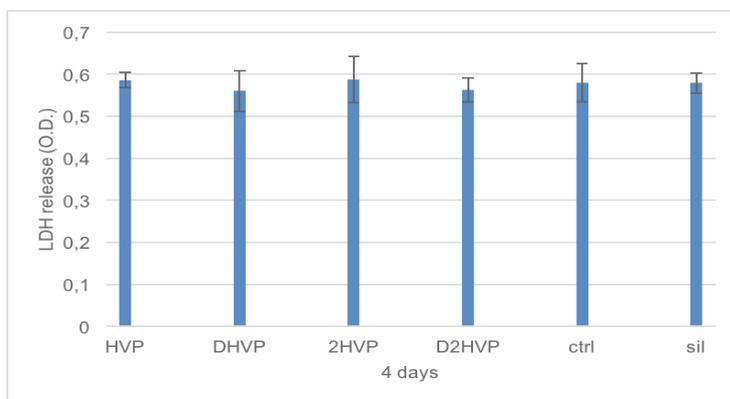


Figure 2: a) H-osteoblast adhesion on functionalized bioceramic foams, determined at 2 hrs from the seeding, through MTT assay. b) H-osteoblast proliferation on functionalized bioceramic foams, determined at 4 days from the seeding, through CFSE probe assay “Sil” sample refers to the silanized scaffolds; “ctrl” refers to a RGD mutated sequences, not showing adhesive properties, while “W/D” are not-treated Wallostonite-Diopside foams. *, ° P-value<0.05 Student’s t-test compared to “Sil” and “ctrl” respectively.

All the samples functionalized with the peptides mapped on Vitronectin show increased cell proliferation with respect to the control and silanized foams: in particular, the functionalization with retro-inverted sequences doubles the proliferation of HVP and 2HVP samples. LDH test demonstrated the absence of cytotoxicity induced by functionalization treatments (Figure 3).



CONCLUSIONS

Adhesive peptide grafting to bioceramic foams enhances h-osteoblast proliferation at 4 days in comparison with samples functionalized with a not-adhesive sequence or only silanized. The proliferation increase is particularly dramatic for retro-inverted analogues (DHVP and D2HVP) and could be due to their capacity to maintain the bioactivity of natural sequences (HVP and 2HVP) jointed with their complete stability to enzymatic degradation.

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RAPID PROTOTYPING OF MULTIMATERIAL NANOCOMPOSITE SCAFFOLDS

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Keywords: scaffold, PCL, nanocomposite, rapid prototyping, superparamagnetic

INTRODUCTION

The tissue engineering approach involves the use of 3D porous scaffolds in order to regenerate a tissue or an organ [1], and additive manufacturing, also known as rapid prototyping or 3D printing or solid freeform fabrication, is a key technology to biomanufacture morphologically controlled, reproducible and custom made scaffolds [2]. 3D fiber deposition, also known as fused deposition modeling, is the preferred additive manufacturing approach for processing highly filled polymer composites [3]. Through this technique a continuous fiber is extruded and deposited from the melt or solution state, and a CAD/CAM system allows to manufacture the 3D physical model layer-on-layer. The resolution of the 3D fiber deposition approach depends on the nozzle/fiber diameter. On the other hand, stereolithography is another additive manufacturing approach which prevents the use of highly filled resins, but it allows a very fine resolution of the scaffold [4]. Through this technique, polymerization occurs via a photocurable process activated by a ultraviolet light reflected by micro-mirrors. Recently, magnetic nanoparticles (MNPs) have been incorporated into poly(ϵ -caprolactone) (PCL) and processed through additive manufacturing approaches [5]. As a result of the superparamagnetic feature of these scaffolds, a more efficient cell seeding and a remarkable bone regeneration have been achieved [6]. Accordingly, the aim of the current research is to biomanufacture multimaterial scaffolds for the

regeneration of osteochondral bone by combining stereolithography and 3D fiber deposition approaches. Morphological and viscoelastic properties are investigated through SEM and DMA. Cylindrical porous scaffolds, based on PEG/MNPs 95/5ww, reproducing the thin layer of cartilage were processed through stereolithography. These scaffolds were properly positioned in the 3D fiber deposition equipment and the composite stratification reproducing the bone scaffold counterpart was carried out using PCL/MNPs 80/20 nanocomposite.

RESULTS AND DISCUSSION

Figure 1 shows the morphology of the multimaterial cylindrical scaffold processed by combining stereolithography and the 3D fiber deposition. An almost constant storage modulus of 4.45 MPa was recorded for the PEG based scaffold between 0.01Hz. Instead, the loss modulus is almost constant up to 1Hz, while it shows a consistent increase from 120kPa to 250kPa through the last two decades of the frequency sweep. The shock absorbing or damping capability of a material is directly related to the phase angle between stress and strain. The phase angle determines the amount of stress that the structure is capable to dissipate, and the loss factor is defined as the tangent value of the phase angle.

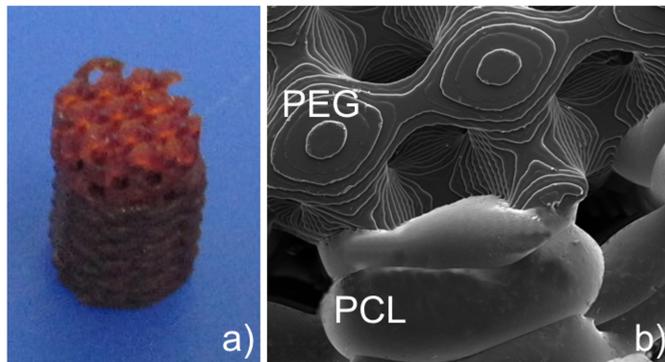


Figure 1: multimaterial nanocomposite scaffold. a) optical imaging of the whole cylinder, b) SEM imaging showing the PEG/PCL interface

The capability of the PEG/MNPs 95/05 scaffold to damp mechanical stress in terms of the tangent of the phase angle is reported in figure 2a. The storage and the loss moduli of the PEG based scaffolds are comparable to those of the articular cartilage.

In fact, the storage and the loss moduli of the articular cartilage varies between 176 kPa and 249 kPa and between 470kPa and 1MPa [7]. Also, the damping properties of the PEG based scaffolds are in agreement with phase an-gle measurements of bovine articular cartilage [8]. The storage modulus of PCL/MNPs 80/20 magnetic scaffolds is almost constant between 0.01 Hz and 10 Hz, while it slightly increases as the frequency in-creases from 1Hz to 100Hz, overall ranging between 100 MPa and 110 MPa.

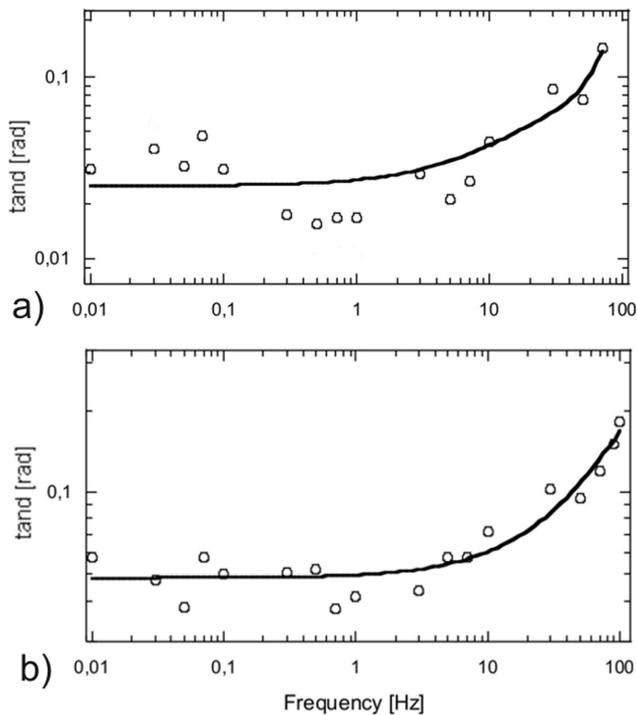


Figure 2: damping capability of thermoplastic scaffolds. a) PEG/MNPs 95/05 scaffold; b) PCL/MNPs 80/20 scaffold.

The loss modulus is almost constant between 0.01 Hz and 1 Hz, while triplicates through the last decade of the frequency sweep, overall spanning from about 6 MPa to 20 MPa. Figure 2b shows the remarkable damping capability of the PCL/MNPs 80/20 scaffold. This feature has to be ascribed to the viscoelastic nature of PCL in the rubbery state at 37°C. It is interesting to observe that the storage modulus of subchondral bone of the human tibial pla-teau increases, in a similar fashion of PCL based scaffold, as

frequency is increased, spanning from 53 MPa to 230 MPa according to the site and frequency. Moreover, the loss modulus of PCL/MNPs scaffolds is similar to those of human bone in the tibial plateau [9]. Hence, the PCLMNPs scaffolds manufactured through 3D fiber deposition mimic viscoelastic properties of subchondral bone.

CONCLUSIONS AND/OR OUTLOOK

By combining stereolithography and 3D fiber deposition techniques it is possible to fabricate a hybrid multimaterial scaffold suitable for osteochondral tissues regeneration. These scaffolds adequately reproduce viscoelastic properties of trabecular bone and of articular cartilage tissues.

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MICROFABRICATION TECHNIQUES FOR TISSUE ENGINEERING

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The advent of microfabrication technologies is rapidly revolutionizing several aspects of tissue engineering.

In the last decade, additive manufacturing (AM) has gathered great relevance for the fabrication of tissue engineered constructs, allowing unprecedented resolution in the deposition of cells and biomaterials. As a further improvement of AM, the introduction of microfluidic-based dispensing heads has enhanced the spectrum of processable materials, and has enabled the high resolution dispensing of cell-laden hydrogels.

Microfluidics has also found an application in the fabrication of scaffolds by exploiting the synthesis of biphasic systems, such as in the case of microfluidic-based foaming/emulsifying of biopolymeric systems. Also in this case, access to microfabrication technologies resulted in improved resolution and control over scaffold microarchitectural features in term of total porosity, pore size and interconnect size, all of which deeply influence scaffold performances.

Another striking example of how microtechnologies have boosted the field of 3D biology is represented by the so-called cell/organ-on-a-chip approach. Here, soft lithographic technologies are used for the microfabrication of cell culture microenvironments, specifically designed to recapitulate the histoarchitectural arrangement and the salient pathophysiological traits of tissues and organs.

The presentation will overview the most remarkable applications of microfabrication and microfluidics in the broad field of tissue engineering and 3D engineered tissue models.

FABRICATION AND PLASMA TREATMENT OF 3-D POLYMER SCAFFOLDS AND THIN FILMS FOR BIOMEDICAL APPLICATIONS

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Keywords: polymer scaffolds, plasma treatment, surface wettability, synchrotron radiation, electrospinning

INTRODUCTION

Electrospun polymer 3-D scaffolds are gaining interest as biomaterials for repairing of damaged bone tissues. The structure of scaffolds during fabrication process depends on many factors; one of them is a type of collector used in electrospinning. To collect charged fibers, collector is fabricated from a conductive substrate and in different shapes. In the case of 3-D scaffolds applied for bone tissue engineering, the distribution of fibers in a whole volume of the scaffold has to be uniform for homogeneous cells adhesion and proliferation. Conventional techniques used for investigation of biomaterial structure provide information only on 2-D level without accessing in-depth information or with low resolution. In this study, an internal structure of polycaprolactone (PCL) polymer 3-D scaffolds obtained with static plate (SP) and

rotating mandrel (RM) collectors were compared by means of high resolution X-ray computed tomography.

Furthermore, plasma modification of 3-D scaffolds using radio-frequency (RF) plasma reactor was carried out to improve hydrophilic surface properties (wettability). PCL, like other synthetic polymers, is less hydrophilic compared to biological tissue surfaces, impeding the penetration of cell into the interior structure. Poor wettability of 3-D scaffold surface leads to poor cell adhesion resulting in poor migration and proliferation of osteoblasts to form bone. Plasma treatment is one of the most perspective method to improve surface biocompatibility of polymers due to formation of hydrophilic functional groups on the surface and improve wettability [1-4].

RESULTS AND DISCUSSION

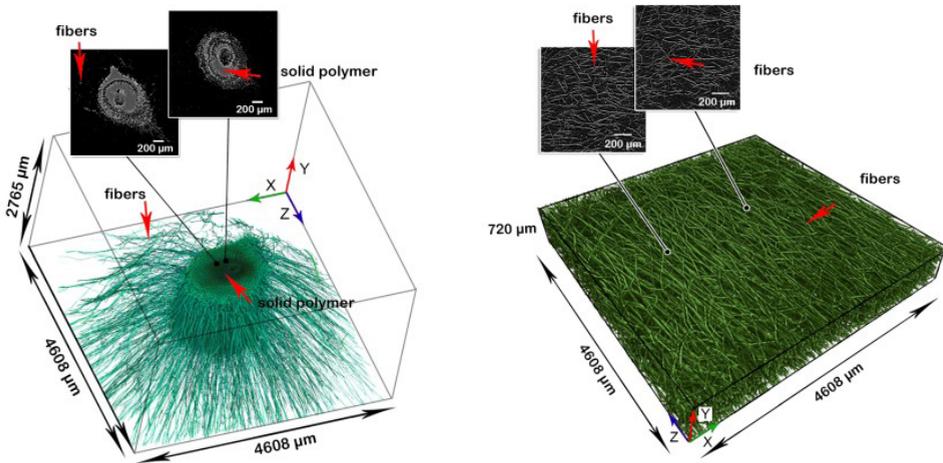


Figure 1: 3-D visualization and 2-D slices from the reconstructed 3D volumes of the **a)** PCL-SP and **b)** PCL-RM samples

A typical result of the 3-D reconstruction of the PCL scaffolds is presented in Figure 1. Comparison of two microstructures revealed that in case of PCL-SP the internal structure included not only the presence of fibre structure, but inhomogeneous polymerized fibres forming a pillar, which is not desired. On the other hand, air voids in the solid polymer part were detected. In case of PCL-RM sample, the formation of micro-fiber structure was uniform in the whole volume of the sample [5].

Surface wettability was assessed by water contact angle analysis and the results obtained are presented in Fig. 2.

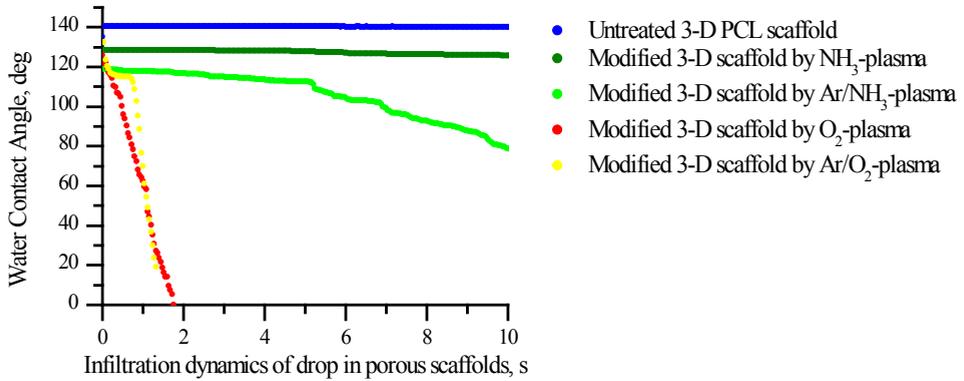


Figure 2: Water contact angles on porous scaffolds as a function of contact time

Untreated 3-D PCL scaffolds revealed to be hydrophobic: contact angles were $136^{\circ} \pm 4^{\circ}$ and water drop did not penetrate into the scaffolds. Plasma treatment did not change the structure and diameter of fibers. Modification of 3-D PCL scaffolds by using oxygen plasma promotes improvement of wettability and water droplets penetrate into porous scaffolds within less than 2 seconds. Ammonia plasma did not improve surface wettability. From our results to improve wettability of 3-D scaffolds one needs to use plasma of a gas mixture of argon with ammonia (Ar/NH₃ - 60/40). However, infiltration dynamics of water droplets into porous scaffolds treated by oxygen or argon/oxygen-plasma are significantly better than after argon/ammonia-plasma treatment.

CONCLUSIONS AND OUTLOOK

The obtained results demonstrate that the rotating collectors allow a uniform fibre structure to be achieved throughout the sample thickness. Synchrotron X-ray computed tomography is a powerful tool to visualize an internal structure of 3-D polymer scaffolds. Plasma treatment of 3-D PCL scaffolds promotes wettability improvement, which potentially result in cell infiltration into the scaffolds.

ACKNOWLEDGMENTS

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3D PRINTING REVOLUTION FOR NANOTECHNOLOGY AND BIOMEDICINE

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Keywords: 3D printing; carbon nanotubes; buckypapers; nanomedicine; tissue regeneration

INTRODUCTION

The 3D printing is about to really transform our lives. While traditional laser and inkjet printers only make marks on paper, 3D printers build up solid objects one very thin layer over another. We are beginning to be surrounded by many prototypes, jewellery, sunglasses, works of art, toys and vehicle parts [1], but the next expected revolution is going to impact on the nanotechnology and biomedical fields [2]. Here we describe how 3D printing has been employed to culture cells on a nanostructured sheet of carbon nanotubes.

RESULTS AND DISCUSSION

Carbon nanotubes can be used to prepare a particular nanostructure, referred

as 'buckypaper', which is macroscopically similar to a sheet of paper. Buckypapers are generally obtained by filtering a mixture of dispersed carbon nanotubes under pressure. To grow cells under this substrate, we first prepared a proper container in a petri dish, able to retain the culture media and allocate a small piece of buckypaper for culturing cells (Figure 1). In fact, buckypapers are hydrophobic and generally float freely upon the culture media if not properly fixed, preventing the cells from adhering to it.

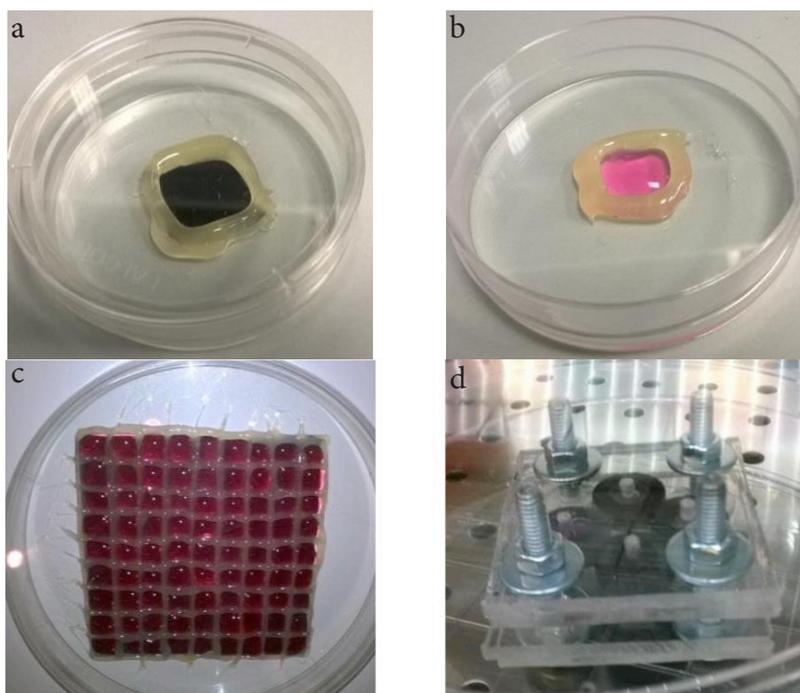


Figure 1: A) Cell culture medium (200 μ l) confined within an artificial well made by hot melt adhesive. B) A sheet of buckypaper has been attached at the bottom of a petri dish for culturing cells. C) A large sheet of buckypaper has been attached to a 'home-made' multiwell petri dish for culturing cells. D) Plexiglass device for culturing cells on buckypapers.

However, these 'home-made' devices are not practical to use, difficult to sterilize, and contamination is not perfectly controllable.

Therefore, we designed biocompatible glycol-modified polyethylene terephthalate (PETG) thermoplastic cell culture devices, which allowed to culture cells in a multiwell format (Figure 2) where the independent wells guarantee sterile conditions.

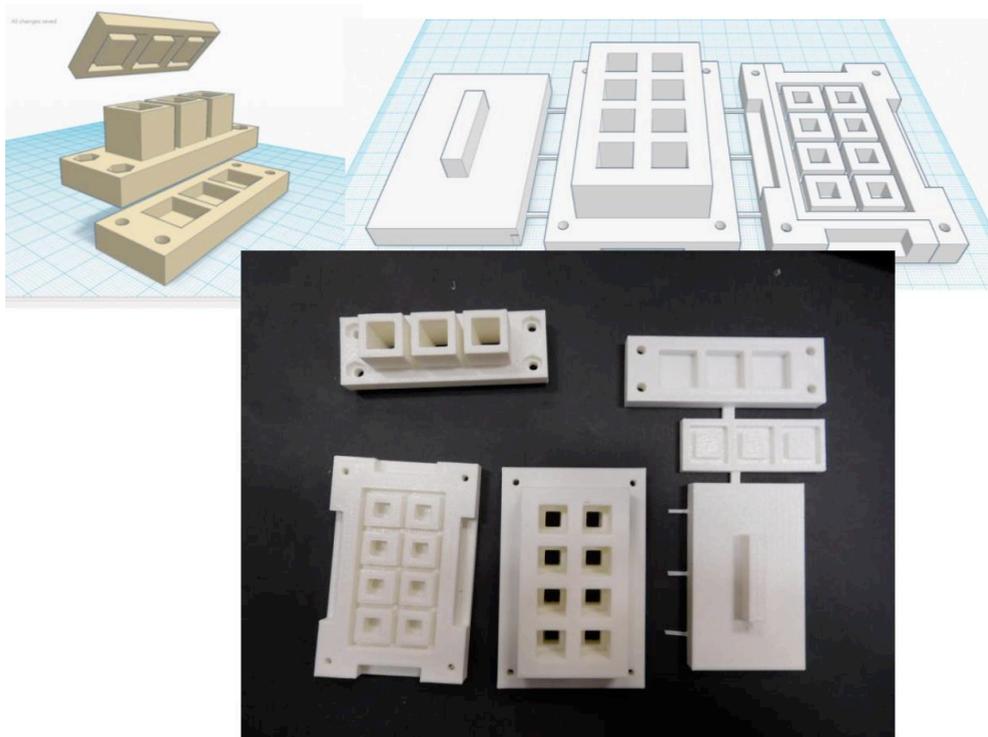


Figure 2: 3D printed devices for cell culture on buckypapers. Sketch of A) three-wells format, B) eight-wells format. C) Real models.

These devices effectively replaced 'home-made' cell-culture devices such as those depicted in Figure 1. Under these particular conditions, cells are able to grow and form monolayers.

Moreover, to validate the use of these substrates as drug delivery vectors, we modified buckypapers by polymer-coating and this functionalisation allowed an easy transduction of cells by a fluorescent model of microRNAs (i.e., small non-coding RNA molecules able to regulate gene expression post-transcriptionally).

We found that the 3D prototyping of a cell culture device gives the opportunity to easily culture cells on buckypapers, avoid contamination and potentially employ these systems for high-throughput screening.

Interestingly, the support that we used (i.e. the buckypaper) is only one of the numerous examples of biomaterials that it is possible to employ in biomedical applications for culturing cells. Therefore, we envisage that in the near future a growing number of materials and potential applications in the biomedical field will appear on the horizon.

CONCLUSIONS

3D printed devices can open new perspectives not only for targeted manufacturing in the biomedical field but also for producing novel materials and 'bio-devices' for nanomedicine applications.

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3D-INK JET PRINTING OF BIOGLASS SCAFFOLDS FOR BONE REGENERATION APPLICATION

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Keywords: calcium phosphate/bioglass scaffolds, bone graft, inkjet powder printing, spray-drying granulation

Bone fractures represent a significant concern due to the increased population life expectancy. When such fractures overcome a certain critical defect size, body induced autorepair cannot restore lost skeleton functionality. Medical treatment involves bone grafting, a common surgical procedure with more than 2.0 million grafting procedures performed worldwide each year. Autologous bone grafts are currently the golden standard treatment but are associated with donor-site complications, risk of infection and size limitations. Artificial bioscaffolds with tailored porosity, architecture and composition present an alternative to autologous grafts and are excellent 3D templates to provide structural support for the newly formed bone. Progress in material science and the better understanding of bone-healing biology resulted in the development of numerous alternative bone graft substitutes, such as calcium phosphates and bioglass products. Furthermore, constant developments of the additive manufacturing techniques in medicine enabled custom-made scaffolds of complex geometries. The combination of bioceramic materials and the powder inkjet printing process enables the fabrication of osteoconductive and osteoinductive scaffolds, mimicking natural bone strength, readily available, patient-specific, cost effective and available in the required amount.

In this work, fabrication of scaffolds was carried out by the inkjet powder process with a commercial 3D inkjet printer. This low temperature printing technique holds great promise in manufacturing bone scaffold substitutes with enhanced properties over traditional techniques and great flexibility in employed materials. The aim of this

study is to investigate the processing and the possible biomedical use of 3D powder-printed tricalcium phosphate/bioglass composite scaffolds for the reconstruction of bone defects. The fabricated scaffolds were computer-aided designed (CAD) with different geometries and pore interconnectivity. Powder feedstock requirements were optimized through the spray-drying granulation process. Control over the co-current spray-drying parameters yielded bioceramic feedstock with optimal granulometry and morphological characteristics. Adhesion of ceramic powders in the powder bed was investigated by both powder coating and powder blending method with a water soluble polymer. Characterization techniques utilised in this study included flowability tests, differential thermal analysis (DTA), scanning electron microscopy (SEM), compressive strength testing and X-Ray diffraction (XRD) phase composition analysis.

IN VITRO AND IN VIVO STUDY OF 3D PRINTED (ALGINATE)-(GELATIN)-(CA-P-MATERIALS) CONSTRUCTIONS FOR BONE DEFECTS REPLACEMENT

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Keywords: 3D-printing, sodium alginate, calcium phosphate, biocompatible materials, bone tissue regeneration.

INTRODUCTION

Development of advanced biomaterials intended to restore bone defects, represents an urgent social and scientific problem due to the incidence of bone tissue pathologies resulted from trauma, tumors or degenerative age-related malformations, and on the other hand – by steady ageing of human population in whole. In terms of bone defects replacement, an ideal decision would be a creation of an individual three-dimensional constructs with high porosity and predetermined structure corresponding to the individual patient MRI data. One of the most perspective approaches to obtain such constructs is to use rapid prototyping, in particular, three-dimensional printing (3D printing) using convenient biomaterials. Thus, synthesis of biocompatible composite materials (“ink”), suitable for 3D printing as well as development of novel techniques for the replacement of especially massive osteochondral defects is a fundamental challenge in modern biomaterial science [1-3].

Given the fact that natural bone comprises a mineral-polymer nanocomposite, we prepared complex printable “ink” consisted of the different biopolymer/calcium phosphate combinations. The aim of this study was to evaluate physical properties

and to perform in vitro and in vivo tests of 3D-printed constructs based on sodium alginate/tricalcium phosphate, sodium alginate/gelatin/tricalcium phosphate, sodium alginate/carbonated hydroxyapatite, sodium alginate/octacalcium phosphate, and sodium alginate/gelatin/octacalcium phosphate. In addition, we developed a new method of 3D-printing using above mentioned compositions and performed complex testing of their mechanical and biological properties [4].

MATERIALS AND METHODS

The methods of 3D constructions producing included 3D printing of components with crosslinking agent (CaCl₂), their freezing, sublimation, "forced shrinkage", and γ -ray sterilization (15 kGr). A structure of 3D constructions, their porosity and strength characteristics were studied. Using scanning electron microscopy it was found that all types of constructions have a lamellar structure of alginate component with spherical inclusions of calcium phosphate. Total porosity of species was 54,5-63,9 %. Calcium phosphates in the constructions reserved initial phase composition. Compressive strength of 3D constructions depended on inorganic component and its concentration and was 1,8-3,7 MPa with ultimate strain 12,3-12,6 %.

Cytocompatibility of 3D printed constructs we studied in experiments with immortalized human fibroblasts (clone I 608 hTERT, Engelhardt Institute of Molecular Biology, Moscow, Russia) and MG-63 human osteosarcoma (Institute of Cytology, St-Petersburg, Russia) cell lines. The dynamics of cell population growth in the presence of constructs was assessed by MTT assay [5]. Biocompatibility of 3D-printed constructs were examined by means of subcutaneous implantation in mice BDF1 and their osteoplastic potency - in the femoral bone defect model in Wistar rats. Animals were sequentially taken out of the experiment in 3, 6, 9 and 12 weeks after surgery and microscopic analysis of hematoxylin-eosin stained histological specimens was performed.

RESULTS AND DISCUSSION

The results of in vitro experiments indicated histocompatibility of all studied compounds in terms of I 608 hTERT fibroblasts and MG-63 cell cultures, which was concluded from the absence of toxicity in the period of 1-14 days coupled with the satisfactory performance of matrix surface properties. So, two weeks cultivation of I 608 hTERT and MG-63 resulted in active colonization of constructs with cells.

Subcutaneous implantation of 3D printed constructs after 14 days resulted in forming a thin transparent connective tissue capsule with a distinct capillary pattern, intimately adjacent to the surface of the constructs. After several weeks capillaries became empty and constructs were almost not visualized. The main markers of constructs biodegradation, which appeared in period of 8-12 weeks after their implantation, included internal rarity and small deformation of structure. The biocompatibility and osteoplastic properties of constructs was further assessed by the integration of the capsule to the surrounding mice tissues. The absence of inflammation reactions at all stages of the experiment, and the active replacement of both alginate and calcium phosphate by immature connective tissue was noticed in all studied groups. The biodegradation of samples was determined by reduction of the calcium phosphate granules size, by the presence of single giant cells and contraction of the capsule.

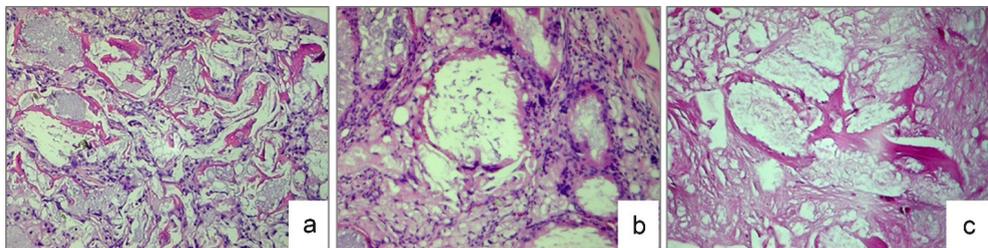


Figure 1: Bone regeneration in rat tibia defect zone after implantation of 3D-printed construct made from sodium alginate + gelatin + octacalcium phosphate. a - 3 weeks, b - 6 weeks, c - 9 weeks after surgery; $\times 100$.

The osteoplastic properties of 4 studied biomaterials - sodium alginate/tricalcium phosphate, sodium alginate/gelatin/tricalcium phosphate, sodium alginate/carbonated hydroxyapatite, sodium alginate/octacalcium phosphate, and sodium alginate/gelatin/octacalcium phosphate were evaluated after in vivo implantation of constructs in rats. The acceptable osteoplastic potencies were observed in all 3D printed constructs. The resorption of constructs after implantation was gradual, meanwhile the rate of biodegradation determined mainly by type of calcium phosphate component. The most active bone formation was noticed in defects, substituted by 3-component implants - scaffolds prototypes based on alginate, gelatin and OCP. At the level of histological examination it was confirmed by: a) the earliest onset of bone formation in the defect area; b) a larger volume of new bone tissue at the same time of observation; c) a larger number of osteoclasts in the area of the defect; d) activation of additional enchondral

mechanism of bone formation together with common periosteal osteogenesis which was observed for other tested compounds.

CONCLUSIONS

In general, all three-dimensional scaffolds confirmed the osteoconductive and osteoinductive features, but on the strength of all the evidence parameters the compounds of sodium alginate + octacalcium phosphate and sodium alginate / gelatin + octacalcium phosphate demonstrated the best potency. The main markers of reparative osteogenesis into the defect area with implanted 3D printing constructs included early beginning (after 3 weeks) of periosteal neoosteogenesis process with the formation of medullary hematopoiesis foci in all studied groups, and the involvement of enchondral bone formation mechanism mainly in groups with gelatin. Altogether received data provides the promising outlook for further improvement of 3D printing and investigations of described 3D constructions as osteoplastic materials. This work was supported by the Russian Ministry of Education and Research Grant (agreement No. 14.604.21.0132 dated 21/10/2014, unique ID: RFMEFI60414X0132).

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3D PRINTING OF SILICA NANOPARTICLE – POLYCAPROLACTONE HYBRID SCAFFOLDS FOR CARTILAGE REGENERATION

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Keywords: Silica Nanoparticle, GPTMS, Hybrids, 3D Printing

INTRODUCTION

This project focuses on making a novel 3D printed hybrid scaffold to be used as cartilage regeneration templates. Unlike conventional scaffolds, a hybrid scaffold is defined as a single phase material which means both of organic and inorganic components interact at the nano-scale. The organic component, usually polymers, provides toughness and the inorganic component, silica network, provides the bioactivity. Because of the molecular level interaction, hybrid scaffolds are able to degrade congruently [1].

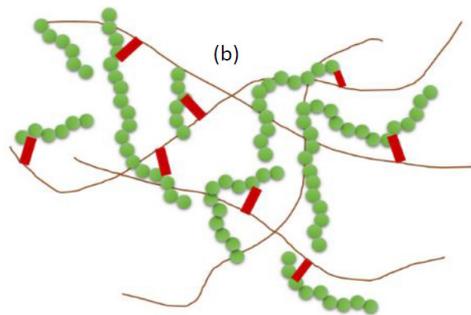


Figure 1: The structure of a class II hybrid

Polycaprolactone (PCL) was chosen to make the organic network owing to its biodegradable property and silica nanoparticles (SNP) were chosen to form the silica inorganic network. Silica nanoparticles were first functionalized with glycidyoxypropyl

trimethoxysilane (GPTMS) and this resulted the hydroxyl group on the original SNPs were replaced by epoxide rings from GPTMS[2]. Followed by the epoxide nucleophilic ring opening, polymer network was covalently connected with SNP inorganic network.

Extrusion printing was chosen as the 3D printing method in this project. Scaffolds will be fabricated layer by layer with hybrid material continuously being extruded out from the printing tip. The structure of scaffolds will be 90° mesh and such alignment of struts can improve scaffolds' compressive strength[3]three-dimensional (3D).

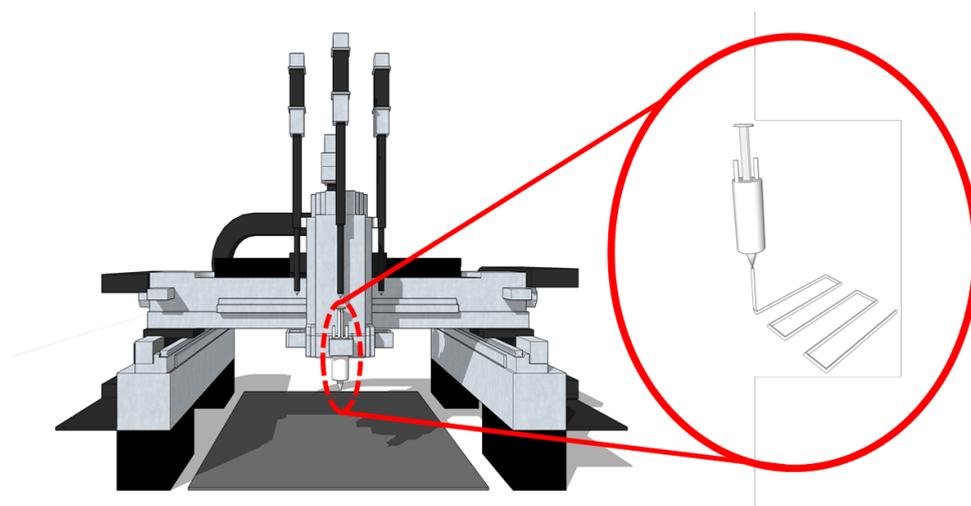


Figure 2: A 3D sketch of the extrusion printer. Hybrid sol ink was loaded into the syringe which then fixed onto the printing head. Continuous filament was fabricated.

RESULTS AND DISCUSSION

The success functionalization of SNP with GPTMS was assessed by TGA (Figure 3a) and zeta potential data. Zeta potential of the bare SNP was -31mV and after the functionalization it went to +30mV. SEM images (Figure 3b) showed that functionalized particles were more aggregated and this might be caused by the chemical structure of the final product (Scheme 1)

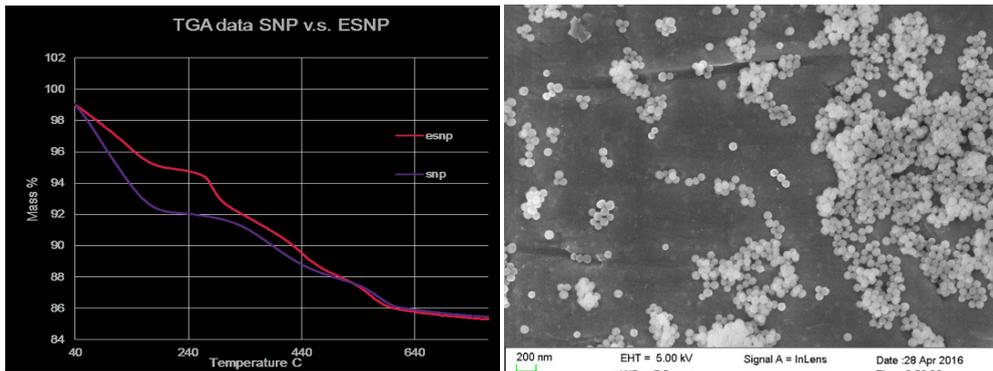
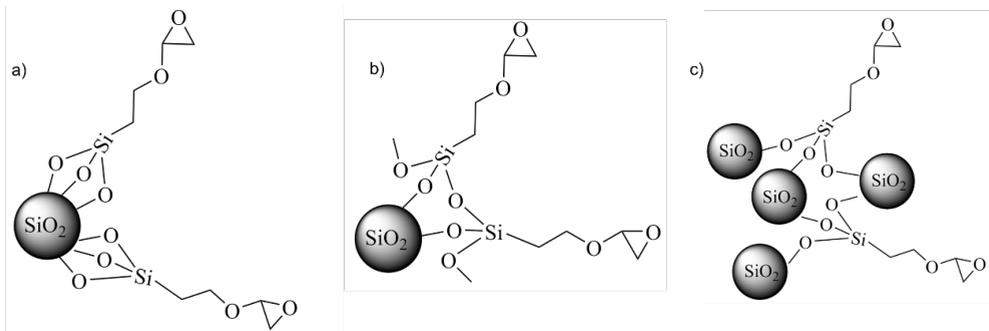


Figure 3: a) The thermogravimetric analysis of bare silica nanoparticles (SNP) and GPTMS functionalized particles (ESNP). b) The SEM image of ESNP.



Scheme 1: Three different chemical structure of GPTMS functionalized silica nanoparticles.

CONCLUSIONS AND FUTURE WORKS

The preliminary result suggested that it is crucial to have SNPs well dispersed in toluene before adding GPTMS in order to reach the desired chemical structure. The synthesis method of bonding functionalized SNP network with PCL network will follow Francesca Tallia's protocol which is undergoing patent work before publishing. The final hybrid product will be characterized by using NMR to investigate the chemical structure. Mechanical test, cell study and dissolution study will be conducted on printed scaffolds to assess the mechanical properties and bioactivities. Impacts on the scaffolds' properties from different organic-inorganic ratio and SNP sizes will also be studied as future works.

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DEVELOPMENT OF 3D PRINTED SILICA-GELATIN HYBRID SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING – EFFECT OF MATERIAL GEOMETRY ON CARTILAGINOUS MATRIX FORMATION

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Keywords: hybrid, 3-D printing, cartilage, tissue engineering

INTRODUCTION

The microstructure of tissue engineering scaffolds such as the pore size has been shown to significantly influence cell behaviour¹. Different cell types, however, require different pore size ranges for optimal growth and matrix formation. Cartilage regeneration has increasingly focused on the concept of “in situ” approach, based on the micro-fracture surgical procedure combined with the use of scaffolds for cell ingrowth and guidance towards tissue formation. Our research group has previously reported the development of silica-gelatin sol-gel hybrid material, which has nano-scale co-networks of inorganic and organic components that allow tailorable degradation rate and mechanical properties². The present study aims to utilise 3-D printing technology to produce silica-gelatin 3-D scaffolds with various structures and, investigate their effects on chondrogenic differentiation.

MATERIALS AND METHODS

The hybrid sol was synthesised using a TEOS based sol-gel process with GPTMS as a coupling agent between gelatin and silica. The rheology of gels with various degrees of cross-linking was examined for optimised 3-D extrusion printing process direct from the sol. Scaffold structure and pore size was controlled by a number

of print file variables, including strut spacing, print head speed, nozzle diameter and material deposition rate and, retained by freeze drying. The 3-D printed scaffolds were characterised by scanning electron microscopy SEM, X-ray microtomography and mechanical tests prior to in vitro biocompatibility screening in accordance to ISO 10993 (Biological evaluation of medical devices). ATDC5 murine chondrogenic cells were seeded onto scaffolds with average pore sizes ranging from 250 to 1000 μm and cultured for up to 21 days. Cell-seeded constructs were analysed for cell attachment, proliferation, cartilaginous matrix formation and expression of cartilage-specific genes and proteins.

RESULTS AND DISCUSSION

SEM and X-ray imaging confirmed the scaffolds contained open and interconnected pores (Figure 1). The Young's moduli of the scaffolds when wet improved significantly to a level comparable to the range of articular cartilage as the pore size reduced to 250 μm .

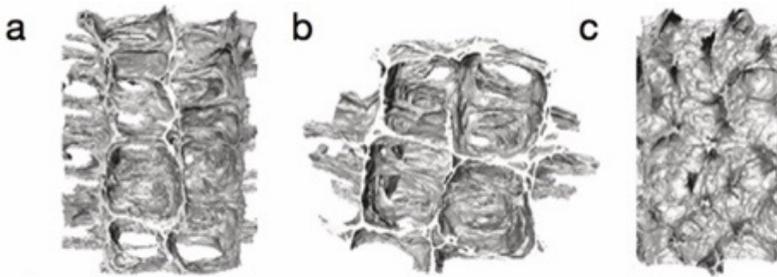


Figure 1. Reconstructed images X-ray images of 3D printed silica-gelatin scaffolds. (a) YZ, (b) XY and (c) XZ view.

MTT metabolic activity assay performed in accordance to ISO 10993 confirmed the silica-gelatin hybrid has excellent biocompatibility. Cytoskeletal constituents Vimentin (intermediate filament proteins) and F-actin (microfilaments) were immunolocalised in ATDC5 cells following 72 hours of cell culture, evidence of functional cell attachment on the scaffolds. WST-1 cell proliferation assay demonstrated improved rate of cell growth in scaffolds with larger pores. Scaffolds with smaller pores up-regulated the expression of hyaline cartilage specific marker Collagen Type II and the amount of sulphated glycosaminoglycan, a major component of cartilaginous matrix (Figure 2).

This was likely due in part to the improved mechanical properties of scaffolds with smaller pores. Further, cells in larger pores, likely experienced environments close to monolayer culture, gradually lost spherical chondrogenic phenotype in a process known as dedifferentiation, evidenced by the up-regulation of fibroblastic marker Collagen Type I. Although cartilaginous matrix formation in scaffolds with large pores was improved by using three times as many initial cell seeding number ($> 10 \times 10^6$ cells), such approach will likely be unrealistic in clinical settings.

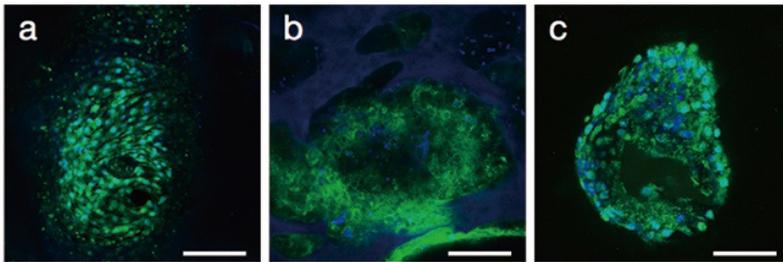


Figure 2: Immunohistochemical staining of day-21 cell-seeded silica-gelatin scaffolds. (a) Sox9, (b) Type II Collagen and (c) Aggrecan.

CONCLUSIONS

Silica-gelatin sol-gel hybrid 3-D scaffolds with tailorable pore size and mechanical properties for tissue engineering applications were successfully manufactured using 3-D printing technology direct from the sol. The results from the present study demonstrated that the size of the space for cell growth is a key factor for cell behaviour and matrix formation for cartilage regeneration.

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3D PRINTING SETS NEW STANDARDS IN MICROFABRICATION

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Keywords: Microfabrication, 3D lithography, scaffolds, tissue engineering, biomimetics

The technique of two-photon polymerization (TPP) allows for high precision additive manufacturing based on 3D digital models with sub-micrometer feature sizes and resolution. In addition, 2D and 2.5D topologies can be fabricated with ultra-high aspect ratios and outstanding design freedom with a resolution between electron beam and UV lithography. This talk gives an overview on the technology and its performance and highlights both scientific disruptive breakthroughs and enabled applications in industry.

The benefits of 3D printing are now fully available on the micrometer scale. While TPP was previously known for ultra-fine yet small objects mostly viewed under the scanning electron microscope, now mm³-scale fabrication, see Figure 1, has become the novel standard in 3D microfabrication with still sub-micrometer features. This closes the gap to conventional stereolithography formerly considered as highest resolution 3D printing technique.

Unique designs and precision open new applications in multiple fields such as photonics, micro-optics, micro robotics, mechanical metamaterials, and life sciences. In life sciences, TPP is used for the fabrication of scaffolds for tissue engineering [1-3], see Figure 2, as well as for cancer cells behavior [4] and for bio-inspired materials that resemble in vivo 3D architectures that influence cell differentiation [5]. Design freedom, resolution, processing speed and a wide range of materials allow to easily produce tailored 3D scaffolds and matrices for mimicking in vivo 3D physiological environments for cell studies.



Figure 1: SEM image of the Taj Mahal demonstrating the fine features enabled by Nanoscribe's high-resolution 3D microprinting.

Filter, mixers [6], complex nozzles [7], micro-robots [8,9] or micro-needles for painless drug delivery exemplify the challenges that can be overcome by 3D printing on the micro- to mesoscale, examples are displayed in Figure 2. And mechanical engineers are enabled to design unique mechanical properties previously unachievable by shaping complex microtrusses. Ultra-light yet strong [10,11] or auxetic [12] materials as well as unfeeling cloaks [13] have been reported.

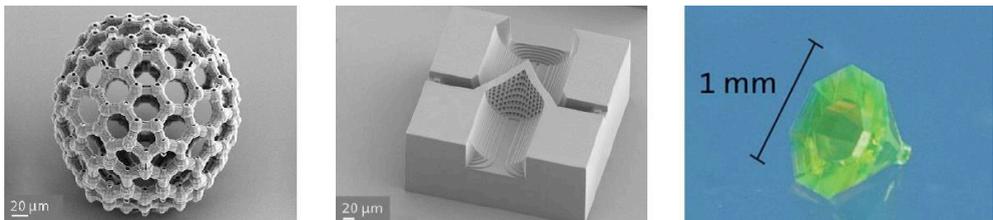


Figure 2: Application examples: (left) Buckyball polymer scaffold that can be used to trap cells inside, (middle) microfluidic filter (design provided by IMSAS) and (right) microfluidic nozzle.

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NOVEL STRUCTURAL CERAMIC COMPOSITES FOR INDIVIDUALIZED 3D-STRUCTURES

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Keywords: structural composites, dense-porous combination, bioceramics, Additive Manufacturing, Freeze Foaming

INTRODUCTION

The objective of the presented work is to develop novel porous, near-net shaped composite structures for personalized bone replacement materials. The suspension-based additive manufacturing (AM) technique Lithography-based Ceramic Manufacturing (LCM) provides a high structural resolution and the manufacturing of dense (> 99%) ceramic components with a high performance compared to other available AM techniques [1]. The commercially available material portfolio for LCM so far is restricted to alumina, zirconia and tricalcium phosphate. That limits the dissemination of this process. Therefore, zirconia and hydroxyapatite as well as mixtures of zirconia and hydroxyapatite were developed to achieve new photo-curable suspension for the used LCM device [2] as door-opener for new applications. On the other hand, the so-called Freeze Foaming process offers the possibility to achieve mainly open porous and interconnected sponge-like structures provably allowing the ingrowth and differentiation of human mesenchymal stem cells [3-5]. Now, the aim of this contribution is to make use of the near-net shaping feasibility of the Freeze Foaming to foam the inner contours of complex LCM-manufactured ceramic shell structures.

RESULTS AND DISCUSSION

As result, we succeeded in the joining of ceramic shell structures with scaffold fillings

to structural composites with dense and porous features in one single 3D structure. That was made possible by according co-sintering steps. First structural combinations led to a demonstrator of a complex-shaped model of a femoral head (see Figure 1) made of zirconia. Computer tomographic (CT) analyses illustrate a mainly partial form and material fit between dense and porous features [6].

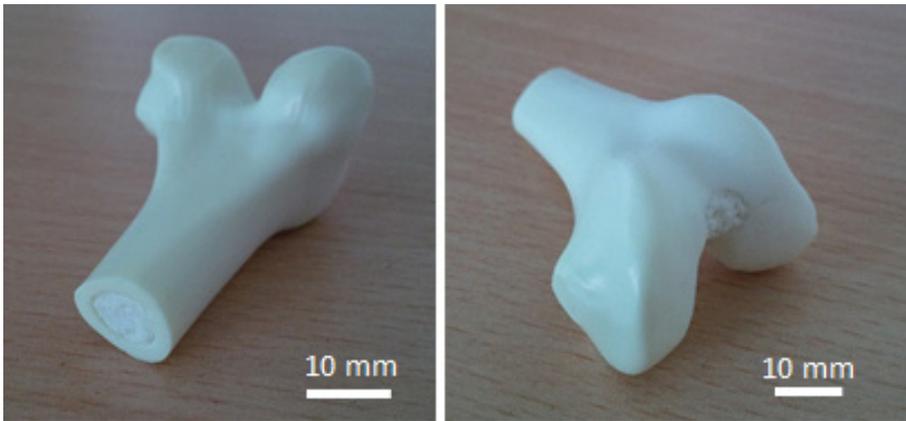


Figure 1: LCM/Freeze Foam demonstrator of a femoral bone model made of zirconia [6]

Zirconia though, is bioinert and remains in the organism. Therefore, the authors progressed towards biodegradable bone replacement material. First experiments with hydroxyapatite (HAp) LCM and Freeze Foam suspensions resulted in test specimen (half-shells and tubes, green-state, see Figure 2) which, according to CT analyses, show a complete material and form fit between dense and porous features after co-sintering at 1350°C (Figure 3).

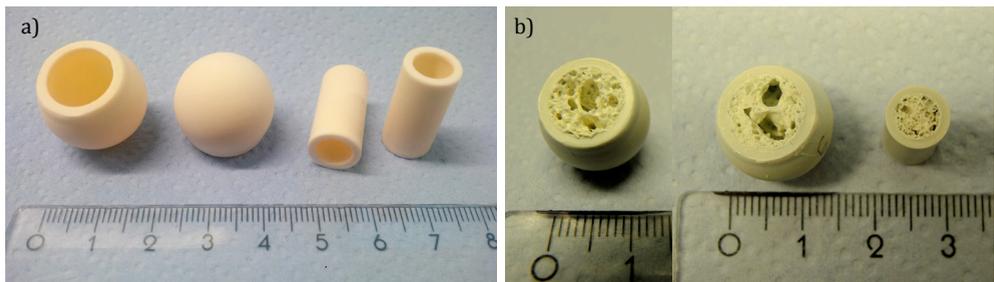


Figure 2: a) HAp green-state specimen of LCM (half-shells and tubes), b) sintered HAp LCM/Freeze Foam specimen

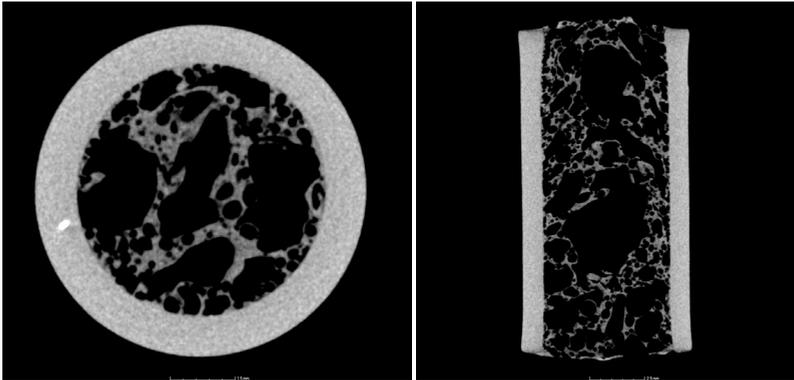


Figure 3: CT analyses of co-sintered HAp LCM/Freeze Foam composite specimen

CONCLUSIONS AND OUTLOOK

Within an oral presentation the authors want to show the potential of the combinational possibilities of Additive Manufacturing and the Freeze Foaming to composites with a new freedom of degree in personalization and therefore, a possible usage in biomedical application such as bone replacement materials. Follow-up research will deal with the strength of the LCM/Freeze Foam interface as well as the overall mechanical strength. Lastly, biological testing do has to show the biocompatibility properties of such new generation of customizable 3D scaffolds.

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INVESTIGATING CELL RESPONSE TO NEW TISSUE ENGINEERING STRATEGIES: RESULTS FROM SYNTHETIC ELECTROSPUN TO NATURAL FIBROUS SCAFFOLDS CULTURES

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Keywords: electrospinning, keratin, tissue engineering, skeletal muscle, bone

INTRODUCTION

Many efforts are dedicated to designing different strategies (i.e. bioengineering tools, microscale/nanoscale biomaterials) to engineer the physical aspects of the natural cellular microenvironment for tissue engineering purposes. Aiming towards this goal, a wide number of synthetic and natural (e.g. collagen) polymers have been used as scaffolds for replacement or repair of injured and diseased organs (1). The use of electrospun aligned fibres mimicking anisotropic structural organization of elongated myofibres is

one of hopeful approach for skeletal muscle repair. In the first part of the present work, we report the results from the investigation of a new micro and nanoelectrospun scaffolds blend of polybutylene 1,4-Cyclohexanedicarboxylate (PBCE) and triethylene cyclohexanedicarboxylate (TECE) polymer as support for muscle tissue regeneration. Besides scaffolds, the physical stimuli (e.g. pulsed electromagnetic field (PEMF), low level laser therapy (LLLT)) represent a strategy to favours a rapid activation of bone tissue repair process (2,3). In the second part, data from the investigation of PEMF application on osteogenesis occurring on keratin substrates, whose unique structure, with controlled-size macro-porosity make them suitable for osteoblast guesing (4), are reported for the first time.

RESULTS AND DISCUSSION

We found that the presence of TECE units in PBCE chain significantly changed scaffolds properties, influencing deeply the in vitro cell response. C2C12 cells adhered strongly to aligned P(BCE-TECE) fibres than to PBCE and film counterparts, aligned themselves in parallel to the direction of the fibrous substratum. Moreover, we observed a better cell differentiation on fibres than to film, as showed by both a higher level of myogenic genes and MHC protein expression. Further, promising results were obtained from preliminary in vivo studies, showing a remarkable interaction between host tissue and implanted-biomaterial. Regarding the osteogenesis on keratin substrates, we compared the cell matrix production and calcified matrix production in un-stimulated and PEMF-stimulated keratin scaffolds, with or without osteogenic factors in the culture medium. After PEMF exposure, in comparison with un-stimulated, we observed in PEMF-treated cultures an enhanced expression of osteogenic markers such as the deposition of extracellular matrix components, calcium and phosphate content (Figure 1).

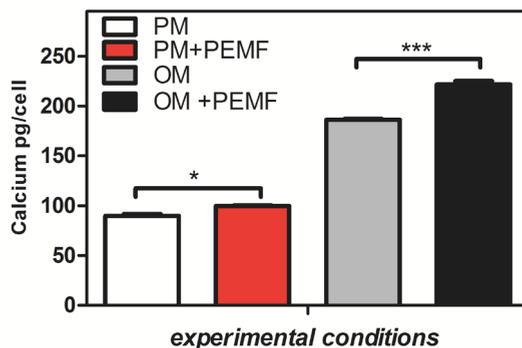


Figure 1: Quantification of Calcium Content. Results are expressed as pg per cell x scaffold and are presented as an average \pm standard deviation (* $p < 0.05$; *** $p < 0.001$). PM = proliferative medium; OM = osteogenic medium.

CONCLUSIONS AND/OR OUTLOOK

In conclusion, we showed the potential use of electrospun PBCE-based scaffolds in skeletal muscle tissue engineering. These newly synthetic substrates mimicking the extracellular matrices signal (micro- or nanofibres), may be useful to explore the influence of ECM on cell behaviour as well to the development of tissue engineering materials for muscle repair. Moreover, we observed the occurring of osteogenesis on wool keratin scaffolds, and its enhancement in combination with PEMF treatment. We further propose that this experiment design may be utilized to stimulate the conversion of bone marrow mesenchymal cells to the osteogenic phenotype on keratin substrates; the resulting data could lead to a potential application in regenerative medicine.

ACKNOWLEDGEMENTS

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CAN ADVANCED HYBRID BIOMATERIALS FULFIL A SURGEON'S CRITERIA FOR LOAD SHARING BIODEGRADABLE SCAFFOLDS IN BONE AND CARTILAGE REGENERATION?

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Keywords: Hybrids; Sol-gel; Cartilage and Bone Regeneration; Bioactive Glass; 3D-printing

MOTIVATION

In 1969, Larry Hench discovered Bioglass, the first osteoinductive material that could not only bond with bone but also encourage regrowth by stimulating the body's natural healing mechanisms. In the last ten years Bioglass has entered common orthopaedic treatments and is even the active ingredient in toothpaste that can treat sensitive teeth. Porous bioactive glasses have potential for bone regeneration applications because they: have an interconnected pore structure suitable for the in vitro growth of blood vessels; degrade; bond to bone; stimulate new bone growth; have compressive strength similar to porous bone [1]. However, they are still not commercially available because they are brittle. For many tissue regeneration applications, toughness is needed.

AIM AND CHALLENGES

In order to overcome this problem, we have developed novel sol-gel hybrids with interpenetrating co-networks of degradable polymers and bioactive silica, with the final aim of creating hybrid scaffolds that act as a single material with tailored and congruent degradation and mechanical properties.

The chemistry and processing is complex to create a successful bioactive hybrid. Main challenges are: achieving a covalent coupling between the interpenetrating organic and

inorganic phases; controlling the degradation rate; tuning the mechanical properties; manufacture the sol-gel hybrid material in a 3D porous shape with the desired level of porosity and interconnections. The polymer choice is a key factor, because polymers must be soluble in the sol-gel process and be functionalised with coupling agents that will bond to the silica [2].

DISCUSSION

In the present talk, an overview of the sol-gel hybrids developed in Jones' group will be presented. From the first generation, which exploited natural organic sources, like polypeptides (e.g. gelatin and poly-g-glutamic acid), and polysaccharides (e.g. chitosan) to the more recent use of synthetic polymers, like polyesters, acrylates and bespoke star-shaped polymers, that can be optimised with a view to the final application.

The broad spectrum of possible alternatives allows the production of several hybrid materials that can be tailored on the basis of the surgeon's needs. Particularly, great efforts are now focused on a new generation of "bouncy" hybrids suitable to restore damaged cartilage. Furthermore, the challenge of introducing Ca and P within the inorganic network at low temperature processing has been overcome, leading to tough and bioactive hybrid materials with excellent potential for bone regeneration. The inherent versatility of the hybrid materials can be combined with cutting-edge processing routes: sol-gel hybrids can be 3D-printed and/or electrospun into bespoke scaffolds that act as temporary templates to guide tissue repair while taking the loads and strains of the body. Advantages of processing routes of 3D-printing and electrospinning will be discussed in comparison with sol-gel foaming and freeze casting.

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PREPARATION AND CHARACTERIZATION OF BIOCELLULOSE COMPOSITES SCAFFOLDS BY IN SITU MODIFICATION OF THE CULTURE MEDIUM OF ACETOBACTER BACTERIA'S USING CARBOXYMETILCELLULOSE.

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Keywords: scaffold, biocellulose composites; in situ; carboxymetilcellulose

INTRODUCTION

Bacterial cellulose (BC) is a natural polysaccharide which has several desirable properties for preparations scaffold materials such as biocompatibility, high porosity, crystallinity, high Young Modulus, elasticity, moldability. Bacteria from the *Gluconacetobacter* genus produce an extremely pure variety of cellulose (free of lignin and hemicellulose) in the form of a highly swollen membrane (around 99% water) in general on the top of culture medium. Microscopically nanofibers can be observed a three-dimensional network of cellulose nanofibers. These nanofibers are responsible for high mechanical, high capacity to absorb skin exudates, wound and burns protection, etc. Pristine BC and BC-based nanocomposites can be also used to produce scaffold for Tissue Engineering Application by *in situ* or *ex-situ* approach. In this work biocellulose composites scaffolds were produced by *in situ* modification of the *Gluconacetobacter* bacteria (ATCC 23769). Culture medium was modified by adding carboxymethylcellulose (CMC) at different degrees of substitution at 1% w/w. BC and BC-CMC samples have been characterized in terms of morpological aspects, mechanical properties, swelling behaviour and sustained release capacity using methotrexate (MTTX) as a drug. Citotoxicity, adhesion and proflerations tests in osteoblastic cells are been in study at this time. BC-CMC biocomposites scaffolds

showed different liquid uptake profiles, so that the BC/CMC 0.7 sample showed the highest liquid uptake (1000%), while BC/CMC 0.9, BC/CMC 1.2 and control (bacterial cellulose only) samples exhibited rates of 100%, 380% and 420%, respectively. These results show that the DS of CMC has direct influence on the BC microstructure, so that molecular structure of the low substituted CMC grade (0.7) arranged in a coiled chain contributes to increase of medium viscosity and thus building a more porous fiber network, which facilitates the liquids absorption. SEM images for all BC-CMC samples show a typical cellulose nanowires network. For the in vitro drug release assay, BC-CMC biocomposites loaded with metformin (MTX) showed a burst effect of release (78%) in the first 15 minutes, for all-prepared samples. Due to the rapid diffusion of MTX molecules that were dispersed between cellulosic fibers, and the extension of release up to 180 min. There was no difference among biocomposites and control sample ($p > 0.05$), but the MTX release from BC/CMC 0.7 was faster than from BC/CMC 0.9 ($p < 0.05$), which is in agreement with liquid uptake results. Tensile strength, elongation at break and Tensile Modulus as shown in Figure 1 below. It can be seen a different profile in function of the different CMC addition. BC-CMC composite sample with higher degree of substitution (DS 1.2) allowed an improvement in the mechanical properties in comparison with pristine BC, which can be related to the greater amount of carboxyl groups in the CMC molecule, and its could be influencing BC biosynthesis process.

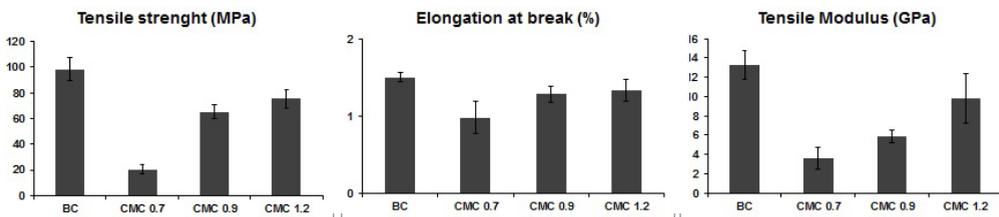


Figure 1: Dynamic Mechanical Analysis (DMA) for pristine BC and all BC-CMC biocomposites

CONCLUSION

BC-CMC biocomposites scaffolds have been obtained by in situ modification. SEM images and swelling results show that DS of CMC has direct influence on BC microstructure, so that molecular structure of the low substituted CMC grade (0.7) arranged in a coiled chain contributes to increase of medium viscosity and thus

building a more porous fiber network, which facilitates the liquids absorption. In vitro assays such as cytotoxicity, adhesion and proliferation cells tests are in developments in order to evaluate BC-CMC composites scaffolf for Tissue Engineering Application.

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TOWARDS ADVANCE HYBRIDS FOR BONE REGENERATION: DESIGNING ACRYLATE POLYMERS WITH PRECISE ARCHITECTURE, COMPOSITION, AND SIZE

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Keywords: Hybrids, Sol-gel, Biomaterials, controlled polymerisation, acrylates

INTRODUCTION

Hybrids possess two different components, commonly an organic and inorganic source, which forms a single phase material through covalent bonding. This nano-scale interaction between the different phases forms a material with synergetic properties. Usually, the organic source provides flexibility while the inorganic phase gives bioactivity and strength[1]. The acrylate polymer and silica containing hybrids have shown promising results in terms of mechanical properties compared to that of the bioactive glass. Specifically, various copolymers of methyl methacrylate (MMA) and 3-(trimethoxysilyl)propyl methacrylate (TMSPMA) containing hybrids have been investigated for bone regenerative materials[2]. However, the copolymers were synthesised through uncontrolled free-radical polymerisation methods in the past studies, which lacked in producing well-defined hybrids. Here, reversible addition-fragmentation chain-transfer (RAFT) polymerisation technique was performed to synthesise various well-defined acrylate copolymers. RAFT polymerisation has also let to produce sophisticated architecture; such as star shape, precisely controlled size and composition of the polymers for hybrids. Furthermore, this study evaluated softer acrylates such as butyl methacrylate (BMA) and methyl acrylate (MA) based copolymers to compare and improve mechanical properties to that of the MMA based hybrids.

RESULTS AND DISCUSSION

MMA based copolymers of poly(MMA-co-TMSPMA) were synthesised in linear (Lin70), randomly branched (Rnd70), and star (Str70) architectures to evaluate the effect to the hybrid's properties. The molecular weight (MW) and TMSPMA molar ratio of the copolymers and silica content of the hybrids were kept constant to set the standardised parameters. The target MW was 60 kg/mol with 9 mol% of TMSPMA, and 70 wt% of polymer source for the hybrid composition. As Figure 1 shows, the hybrids were much flexible and tougher than 70S30C bioactive glass. More importantly the polymer architecture had an impact in mechanical properties. The most noticeable difference was seen in yield stress and compressive modulus values. Yield stress of Str70 was nearly half of Rnd70 and Lin70. Also, compressive modulus of Str70 decreased by approximately 1.6 fold compared to the other hybrids.

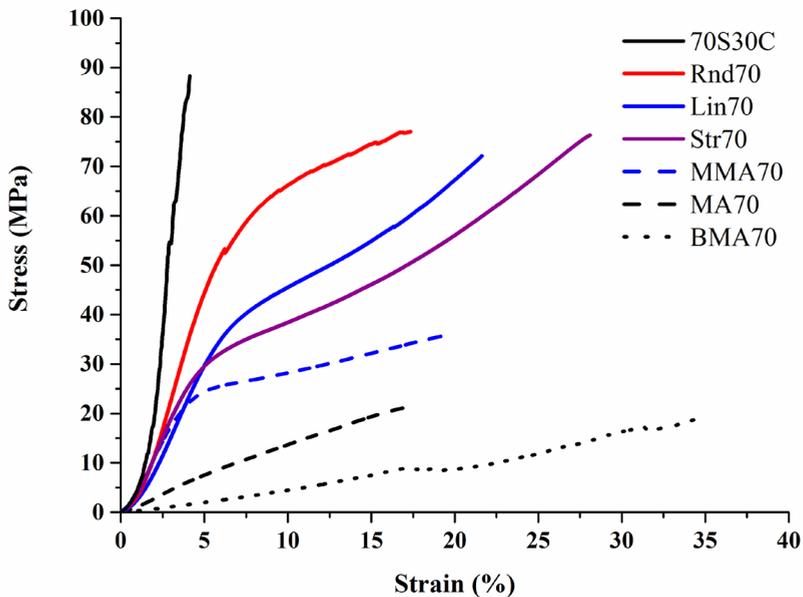


Figure 1: Representative uniaxial compression test curve of the hybrids and 70S30C bioactive glass.

Softer, or low glass transition temperature, acrylate copolymers of poly(BMA-co-TMSPMA), and poly(MA-co-TMSPA) were synthesised to compare the mechanical properties to that of the MMA based hybrids. As expected, softer acrylate containing

hybrids (MA70 and BMA70) displayed different compressive behaviour compared to MMA70. There was absence of the yield point and plastic deformation. Particularly, BMA70 had excessive strain to failure value of 33% despite of having 30 wt% of silica content. Another interesting finding was the difference between MMA70 to Lin70. Both hybrids contained poly(MMA-co-TMSPMA), however with the MW difference of 4 fold. The ultimate stress and compressive modulus values of MMA70 were reduced by nearly 2 fold compared to Lin70, while yield strain and ultimate strain values were very similar. This was possibly due to the lower MW of MMA70, which decreases entanglement of the polymers.

CONCLUSIONS AND/OR OUTLOOK

Various acrylate-silica containing class II hybrids with different polymer architecture, composition, and size were produced. This was possible by using controlled polymerisation technique. From this study, mechanical properties of the hybrids were modified by introducing different polymer architecture. MW of the polymer was also shown to influence the mechanical properties. Designing precise and well-defined polymers opens up more possibility to produce advance hybrids for bone regeneration.

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CARBON NANOTUBE REINFORCED POLY(ETHYLENE GLYCOL) HYDROGELS FOR BONE TISSUE ENGINEERING

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Keywords: bone tissue engineering, carbon nanotubes, hydroxyapatite, poly(ethylene glycol), hydrogels

INTRODUCTION

Treatments leading to improved bone formation are in demand clinically, as millions of patients suffer from insufficient bone healing resulting from traumatic injuries, aging and cancer. The need for synthetic substitutes that mimic the extracellular matrix of bone in terms of composition as well as in structure pushes research towards sophisticated composite scaffolds that not only serve as matrix for cells but also influence favorable cellular responses by incorporation of nanosized features. Bone tissue is an inorganic composite material that is composed of two primary nanophases: collagen type I fibers and hydroxyapatite (HAp). The HAp ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) nanocrystals are deposited in a staggered fashion along the collagen fibers [1-3] through a mineralization process by cells. Carbon nanotubes (CNTs), a macromolecular form of carbon, are known for their unique physical, mechanical and electrical properties which makes them of potential interest for tissue engineering and regenerative medicine applications, and they are believed to mimic collagen due to their fibrillar shape and similar dimensions [4-8]. As hydrogels are being increasingly used in bone tissue engineering (BTE) to serve as a matrix for cells and to promote bone growth, we have engineered hydrogels based on poly(ethylene glycol) (PEG), an interesting synthetic polymer as its chemistry is well-defined [9]. PEG is a good candidate to partially mimic the

characteristics of the extracellular matrix (ECM) of bone, as it is resistant to non-specific protein absorption and absorbs a lot of water when crosslinked. However, the lack in mechanical properties for hydrogels has prevented them so far in giving proper support in bone replacement. In this study, different weight percentages of CNTs were incorporated within a PEG hydrogel to increase its mechanical strength.

MATERIALS AND METHODS

First, hydrogel composites were prepared with pristine multiwall CNTs. Vinyl sulfone functionalized 4-arm PEG (20 kDa) was dissolved in a polyvinylpyrrolidone (PVP) solution (1.5 wt.%, pH 8) and crosslinked via a Michael-type reaction with a di-thiol crosslinker. The preparation of PEG hydrogels with 0.001, 0.025, 0.25 and 0.35 wt.% of pristine CNTs (purity > 95%, $\varphi=9,5$ nm, $L=1,5$ μ m) was done by replacing part of the PVP- solution with a 1 wt.% solution of CNTs (Figure 1A, B). Complete crosslinking was carried out at 40 °C for 70 min, under humidified conditions. To determine the swelling ratio, the hydrogels were put in distilled water and weighed at \times time points and room temperature. After this, the samples were lyophilized, and the dry weight was measured. For rheology, 8 mm diameter hydrogels were prepared and swollen in distilled water. A frequency sweep (angular frequency range [rad/s]: 100 – 0.1 and strain amplitude [%]: 0.1) was executed using a stress controlled rheometer (Physica MCR501) under humidified conditions. Additionally, unconfined compression tests were performed on swollen hydrogels at room temperature by Dynamic Mechanical Analysis (Q800 T.A. Instruments) with a preload of 0.001 N and a strain rate of 5 %/min up to 40 %.

RESULTS AND DISCUSSION

An average swelling ratio of 47.86 (± 2.53) was measured for PEG hydrogels without CNTs, and adding pristine CNTs up to 0.025 wt.% did not show significant changes in swelling, which indicates CNTs in this concentration range do not disrupt the crosslinking density. Adding 0.25 and 0.35 wt.% of CNTs significantly increased the swelling ratio, which indicates the crosslinking density and hydrogel network structure were affected. The addition of 0.025 wt.% pristine CNTs significantly increased the storage modulus (G') of PEG hydrogels from 1420 (± 169) Pa to 1997 (± 85) Pa ($p < 0.05$) (Figure 1C). This means that the addition of 0.025 wt.% CNTs increased the rigidity of the hydrogels. However, adding 0.25 CNTs did not significantly improve G'

compared to the 0.025 wt.% CNT hydrogels ($p > 0.05$). 0.35 wt.% did increase the G' , but not as drastic as initially expected compared to the 0.025 wt.% CNTs. The same trend was seen with the compression modulus, which increased from 3 kPa to 6 kPa when adding 0.01 wt.% CNTs ($p < 0.001$). Additionally, increasing the CNT wt.% up to 0.2 did not further increase the compression modulus. This can potentially be explained by the formation of CNT aggregates, which would introduce stress concentrations.

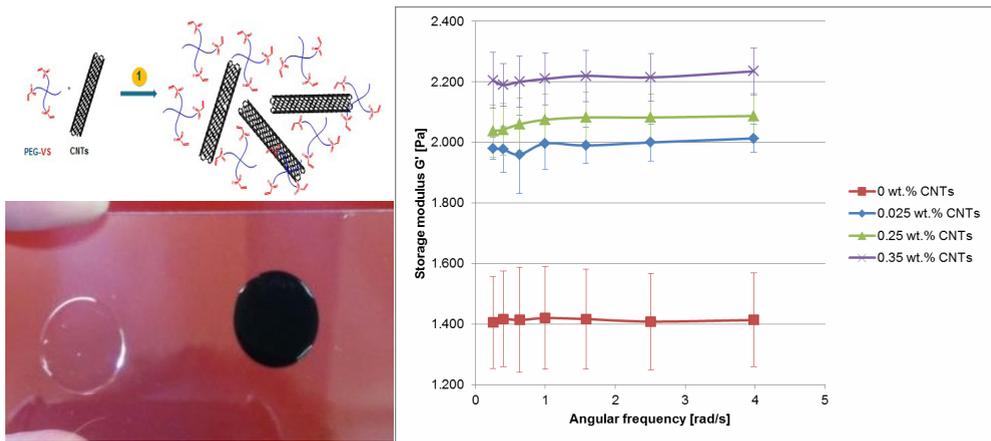


Figure 1: (A) Schematic showing of Pristine CNTs dispersed in PEG network, (B) Pure PEG hydrogel (left), 0.025 wt.% CNT PEG hydrogel (right), (C) Frequency sweep of PEG hydrogels with varying wt% pristine CNTs

CONCLUSIONS AND OUTLOOK

In this study, nanofeatured composites with a high potential for future use in bone tissue repair were created. Hydrogels with 0.01 and 0.025 wt.% pristine CNTs showed an increase in compression and storage modulus, respectively, without disturbing the crosslink density and hydrogel network structure. As future work, the pristine CNTs will be replaced by hydroxyapatite (HAp)-coated CNTs in the hydrogel formulations, which should lead to lower cytotoxicity and induce bone formation due to the osteoinductive properties of HAp [10]. In vitro evaluation of both pristine and HAp-coated CNT reinforced hydrogels will be performed by seeding the hydrogels with NIH/3T3 fibroblast and pre-osteoblast cells to confirm this hypothesis.

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NEAR FIELD ELECTROSPUN FIBRES FOR NEURAL STEM CELL DIFFERENTIATION

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Keywords: near-field electrospinning, nano-fibres, neural stem cells, topography, stiffness, carbon nanotube, neural electrode, thermo-responsive polymer, coaxial fibres, protein delivery, microfluidics, neural migration

INTRODUCTION

Topography and mechanical stiffness of the substrate has been shown to induce neural stem cell differentiation. Herein, near-field electrospinning (NFES)¹ was employed to provide a topographically aligned polymeric fibrous scaffold for neural stem cell differentiation, which mimics the similar process found in the brain cortex and the peripheral neurons. The system could be utilized as a model to study the effect of fibres' sizes, spacing, and stiffness on neuronal differentiation up to 8 days. Unlike conventional 3D aligned fibres, 2D NFES fibres allow progressive imaging of the cells grown on top of the fibres to study various differentiation parameters such as neurite length and the number of neurites per cell continuously. The expression profile of neuronal marker, Map2, could be used to evaluate how much the stem cells have differentiated fully into neurons in different scenarios. The NFES polymeric fibres could be co-electrospun with carbon nanotube to build an electrode which stimulates neuronal stem cells differentiation or composited with thermo-responsive polymer to build an assay-based micro-reactor. Coaxial fibres for sustained protein delivery can also be patterned inside a microfluidics to study neural migration and differentiation along the aligned nanofibres.

RESULTS AND DISCUSSION

- The NFES PEO fibres 'sizes (<2.5, 2.5-12.5, >12.5 μm , measured using an AFM) affect both the neurite length and the number of neurite of neural stem cells. Larger fibres sizes were found to increase the number of neurites and decrease the neurite length in the early days, where the difference become smaller as the cells grow closer to 8 days. Map2 expression profiles show smaller fibres size giving rise to higher proportion of neurons compared to differentiating neural stem cells, however, only the fibres with the sizes similar to conventional size of the neurites (2.5-12.5 μm) could give rise to fully mature neurons.
- The NFES PEO fibres' spacing (50, 100, and 150 μm , measured under light microscope) was found to affect the number of neurites but not the length. The number of neurites of different spacing was found to increase in step-wise manner according to the time it reaches adjacent fibres. Map2 expression profile shows that, with large spacing, the neurite was restricted only one neuron and proportion of mature neurons with high Map2 expression is low, while in small spacing, the proliferation dominates differentiation and the proportion is also low.
- The knowledge of neurons with single neurite on top of the fibres could be found in relatively small fibres with large spacing allow us to study the effect of fibre stiffness on single neurite. Different percentages of PEO and its crosslinker gives rise to fibres with different Young's modulus (3.6, 27, 117 kPa, measured by rheology measurement). It is ultimately shown that fibres with higher stiffness give rise to neurons with higher neurite lengths in early days. However, these differences become less pronounced as neurons grow up to 8 days.
- Single walled carbon nanotube had been successfully co-NFES with PEO, which showed the conductivity of 0.015 Sm^{-1} . Neural stem cell grown on top of the fibres exhibited as high biocompatibility as normal PEO fibres. The PDMS based electrode which was flexible and transparent was built using carbon ink as a connection to a power supply. The sinusoid current waveform at 1.5 mA at 80 Hz was provided to stimulate neural stem cell differentiation. The effect of electrical stimulation on neural stem cell differentiation will be further investigated.
- Thermo-responsive polymer, poly (diethylene glycol methyl ether

methacrylate), or PDEGMA, was blended with gelatine to NFES composited fibres. Neural stem cells grown onto the fibres were shown to adhere to the side of the fibres and differentiated into neurons. The composite fibres showed the LCST of 22 °C, where the polymer chain underwent transition from globule to coiled structure upon temperature drop. 84% of neural stem cells were shown to detach from the fibres and become rounded. The fibres could be patterned inside a microfluidics to grow and collect differentiated neurons for high-throughput assay.

- Coaxial fibres were patterned using NFES technique. The diameters of the core and the shell could be adjusted according to the sizes of the spinnerette and the capillary inside. The use of coaxial fibres allowed sustained release of the protein or drug embedded inside the core of the fibres. Coaxial fibres can be patterned to incrementally increase the spacing, which allows the content inside to release in gradient manner. The patterned fibres could be incorporated inside a microfluidics that can encapsulate collagen gel on top of the fibres. This gel allows the neural stem cells seeded inside to grow, differentiate and migrate along the gradient of the released migration factor. NFES gelatine fibres could be NFES onto this gel, where the neural stem cells could be grown and migrate on top. The system could be used as a model to study different migration systems inside the brain as well as to test the drug upon neural migration and differentiation.



Figure 1: A) Neural stem cells grown on NFES PEO fibres immune-stained with anti-MAP2 (red), Phalloidin (green) and Hoechst stain (blue). B) NFES PEO-CNT fibres in an electrode setup. C) Neural stem cells grown on NFES thermos-responsive PDEGMA-gelatine fibres. D) NFES coaxial PEO fibres with protein-filled core. E) Microfluidics setup incorporating NFES coaxial fibres with incrementally increasing spacing for gradient protein release to study neural migration and differentiation

CONCLUSIONS

PEO, PEO-CNT, gelatine, gelatine-PDEGMA, and coaxial PEO fibres were successfully

NFES which gives rise to different applications to assist neuronal differentiation. NFES fibres' sizes, spacing, and stiffness affect neuronal differentiation in different aspects including the neurite length, the number of neurites per cells, and the expression of neuronal marker, MAP2.

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A NEW MICRO/NANO HYBRID SUBSTRATE FOR NEURONAL NETWORK FORMATION

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Keywords : Micropillar Array, Nanofiber, Neuronal Cell Culture.

INTRODUCTION

The development of a three-dimensional substrate for the in vitro modeling of mammalian brain is based both on the chemistry and on the physical properties of the material of choice. In fact, the natural environment of neurons, i.e. the extracellular matrix (ECM), presents molecular cues in the micro-nano scale that can be mimicked by particular patterning of biocompatible polymers [1]. Recent studies focused on recreating in vivo - like architectures as reliable models for the study of neurodegenerative diseases and for on-chip investigation of cell-drug interactions [2]. In general, cell microarrays are fabricated by a variety of sophisticated techniques such as photolithography, e-beam writing and focused ion beam machining. In this work, we propose a more reproducible method to fabricate a micro/nano hybrid substrate for the formation and growth of neuronal networks. Based on our previous studies [3], the experimental protocol is three step and involves photolithography, hot embossing and electrospinning.

RESULTS AND DISCUSSION

The fabrication of the new micro/nano hybrid substrates is summarized in Figure 1. First, a photoresist mold was fabricated via photolithography. A 25 μm thick negative

resist layer (SU8-3025) was spin-coated on the Cr mask which was patterned with periodic holes by a micro pattern generator (μ PG101). Following by back-side UV exposure, a mold of 25 μ m height pillar array was obtained with 5 μ m diameter and 20 μ m spacing (Figure 1a). Second, the master pattern was replicated into polydimethylsiloxane (PDMS) by soft lithography and used as a negative mold for the second replication step, when the final microarray was obtained by curing an additional layer of PDMS (Figure 1b). Third, nanofibers of poly lactic-co-glycolide acid (PLGA), a biodegradable polymer, were electrospun on the top of the micro PDMS supporter to form a monolayer with pore size ranging from 10 to 15 μ m (Figure 1c). A first test of the newly fabricated substrates was performed using the human glioblastoma cell line U87. A preliminary plasma treatment was necessary to reduce the hydrophobicity of the substrates and enable cell's attachment. Fixation and SEM imaging of U87 cells were performed 48 hours after plating. A PDMS micropattern without nanofiber's coating was used as a control. On the PDMS/PLGA substrate, a higher number of adherent cells (Figure 4a-c) and an increase in cell body' area (Figure 4b-d) were observed comparing to the control.

In a subsequent test, primary hippocampal neurons were plated on the PDMS/PLGA substrate at a density of 300,000

cells/sample. In addition to plasma treatment, a protein coating was performed before cell seeding in order to increase neuron's attachment. Fixation and staining with the neuronal marker α -tubulin I (red), astrocytic marker GFAP (green)

and nuclear marker Hoechst (blue) were performed 48 hours after plating (Figure 4e-f). Neuronal attachment and survival were comparable between the PDMS/PLGA substrate and the standard cell culture on glass coverslip (figure not shown).

We can conclude that the fabrication of the hybrid PDMS/PLGA is a highly reproducible method for producing a

micro/nano architecture for different types of cell cultures. The intrinsic hydrophobicity of the materials used, that often represents an issue for adherent cell culture's applications, was overcome by appropriate pre-treatment of the substrate. Further experiments are necessary in order to analyze the functionality of cells grown on the hybrid substrate.

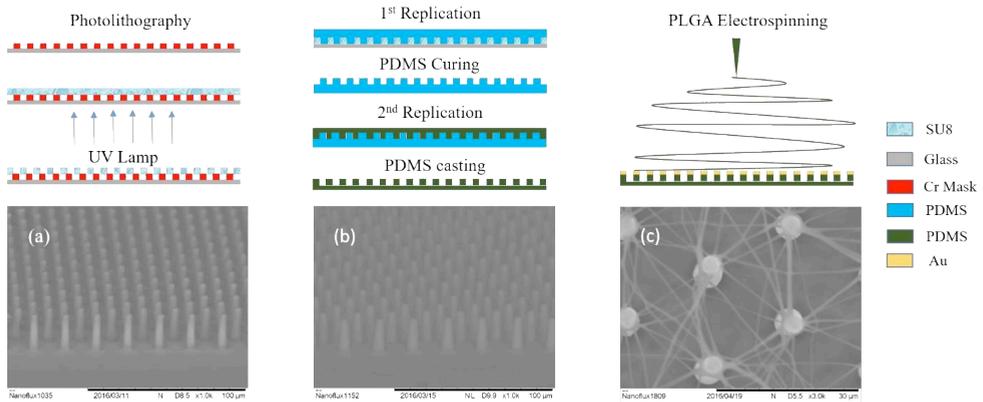


Figure 1: Schematic process of fabrication of hybrid substrates via three steps. I Photolithography. II Soft lithography and casting/hot embossing. III Electrospinning. SEM images show the results of (a) SU8 mold of pillar array, (b) PDMS pillar array supporter, (c) Final hybrid substrate with PLGA nanofibers on top of micropillars.

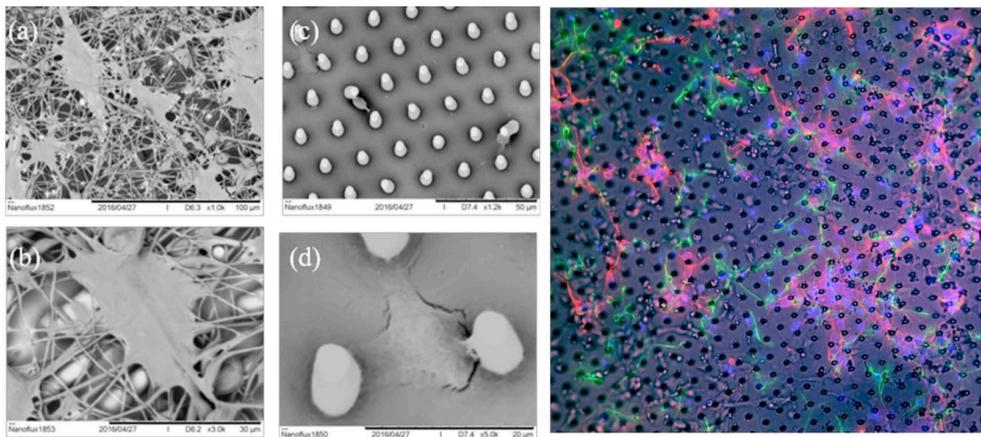


Figure 2: Cell imaging 48 hours after plating on PDMS microarrays. SEM images of U87 cells on PDMS/PLGA (a,b) and on PDMS (c,d) microarrays. Immunofluorescence (e) and bright field (f) images of neurons (red) and glial cells (green) on PDMS/PLGA microarrays.

CONCLUSIONS AND OUTLOOK

These preliminary results show that the new hybrid substrate provides the necessary

features to be considered as an artificial brain's ECM, i.e. three-dimensionality, micro/nanometric cues and biocompatibility. The PDMS/PLGA substrate allows attachment, survival and growth of neuronal cell's types. Next experiments will investigate the spontaneous activity of the neuronal network grown on the micro/nano pattern via calcium imaging techniques, in order to complete the validation of the substrate as a tool for in vitro neuronal modeling. Finally, neuronal networks with different ratios of inhibitory vs excitatory neurons will be grown on the substrate in order to create in vitro models of neurodegenerative diseases and study their properties.

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RESORBABLE DRUG DELIVERY VEHICLES FOR LOCAL TREATMENT OF BONE TISSUE PATHOLOGIES

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Keywords: osteomyelitis, osteoporosis, poly(L-lactide-co-glycolide), drug delivery

INTRODUCTION

Bone infections resulting in osteomyelitis require prolonged antibiotherapy for at least 6 weeks; drugs are usually administered via parenteral route [1]. However, prolonged administration of antibiotics may cause serious side effects such as nephrotoxicity, ototoxicity and may provoke development of antibiotic-resistant bacterial strains [2]. Other bone tissue pathologies such as osteoporosis, bone metastasis or Paget's disease - causing deterioration of bone tissue matrix - are also treated systemically via oral administration of bisphosphonates (e.g., sodium alendronate - ALN) or parenteral administration of calcitonin (CALC). Nevertheless, systemic administration of ALN beside low bioavailability (<1% when administered orally) evokes numerous side effects such as fever, esophageal erosions, ulcers and problems with gastrointestinal tract [3]. Likewise subcutaneous route of administration of CALC brings to the patient many side effects: headache, flushing, nausea and diarrhea [4].

Thus, we propose biomaterials to treat skeletal tissue diseases which are based on micro- or nanospherical carriers assuring controlled release of drugs and site-specific administration. To this end we entrapped drug molecules into long biodegrading poly(lactide-co-glycolide) (PLGA) spherical micro- and nanoparticles. Such loaded vehicles were then processed in a way to obtain different forms of biomaterials (injectable or implantable) that meet the requirements of complex therapies of bone tissue pathologies (Figure 1).

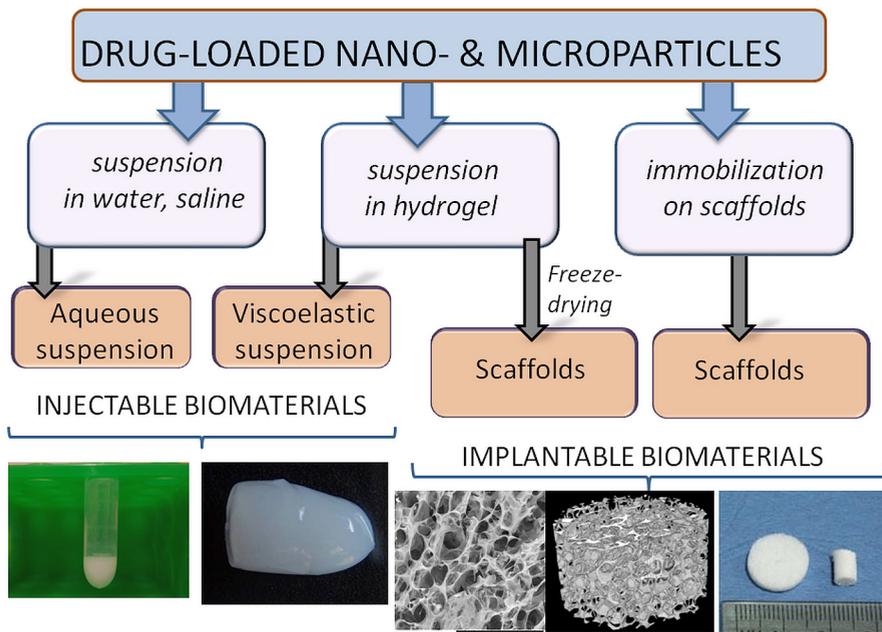


Figure 1: Drug-loaded nano- & microcarriers as a platform to produce injectable and implantable biomaterials for the local treatment of bone tissue diseases

MATERIALS AND METHODS

PLGA micro- and nanoparticles were produced by double emulsification. Their morphology (AFM, optical microscopy), physicochemical properties (size, ζ potential, drug solubilization) and drug release kinetics were assessed. Then the carriers were suspended in hydrogels of protein or polysaccharide origin intended to be administered by injection directly to affected area in bone tissue. The second approach was based on freeze-drying of spherical carriers/hydrogel composites to produce implantable spongy forms of biomaterials, while the third aimed to immobilize the carriers within highly porous PLGA scaffolds.

The systems were studied with respect to surgical handiness, mechanical properties (compression tests, rheology) and drug release kinetics. The systems delivering antibiotics (gentamicin, vancomycin) were tested with *Staphylococcus spp.* via Kirby-Bauer method, while those loaded with ALN and CALC with cells responsible for bone remodelling (osteoblasts and osteoclasts).

RESULTS AND DISCUSSION

The results showed that by changing shear rate in the second step of emulsification it was possible to obtain drug-loaded PLGA particles of defined size (micro- or nanometric) [5]. The carriers released the drugs in a sustainable manner, which was even prolonged when the carriers were suspended in hydrogels or processed into implantable composites [6-8]. The systems containing antibiotics showed antimicrobial activity against classical strains of *S. aureus* and *S. epidermidis* and clinical strains isolated from infected joints [5-7]. The systems containing ALN [8] and CALC downregulated osteoclasts and simultaneously did not affect osteoblasts functions.

CONCLUSION

Proposed processing methods preserved biological activity of encapsulated drugs and thus the systems may constitute a promising solution in site-specific therapies of bone tissues pathologies.

ACKNOWLEDGEMENTS

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EFFECT OF DENSE STRONTIUM CONTAINING BIOACTIVE GLASS NANOPARTICLES ON MACROPHAGES *IN VITRO*

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Keywords: Bioactive glass nanoparticles, Sol-gel process, strontium, macrophages,

INTRODUCTION

Bioactive glass microparticles are widely used clinically as bone graft substitutes for bone and dental implantation. To provide an injectable alternative, the bioactive glass nanoparticles (BGNPs) have been developed. Strontium (Sr) has reported to be use in osteoporosis treatment because its ability to increase bone density. Our previous study showed that new monodispersed dense sol-gel derived bioactive glass nanoparticles containing strontium (Sr-BGNPs) fabricated using a modified Stöber process [1] and their dissolution products have the positive impact on stimulating the osteogenic response of preosteoblast cells (MCT3T-E1) and human mesenchymal stem cells (hMSCs). However, injection of Sr-BGNPs at a bone-healing site might cause foreign body reactions to occur by macrophage cells [2]. Therefore, understanding the interaction of macrophages with Sr-BGNPs is an importance factor for assessing the possibility of using these particles for bone healing. The aim of this research was to evaluate the effect of Sr-BGNPs on macrophages isolated from mice murine bone marrow.

RESULTS AND DISCUSSION

Macrophages were incubated with 0 – 750 $\mu\text{g}\cdot\text{ml}^{-1}$ Sr-BGNPs, which had amorphous

spherical form with 90 ± 10 nm in diameter, for 24 hours prior to evaluating metabolic activity using WST assay (Figure 1 (a)). Macrophage metabolic activity increased at all particle concentrations. Interestingly, a significant increase of cell metabolic activity was observed 25% Sr substitution on a mol basis (25% Sr-BGNPs) in 90 mol% SiO_2 and 10 mol% CaO. Total DNA concentrations of macrophages were not significantly different compared with those treated under basal condition with Si-NPs and Sr-BGNPs at $200 \mu\text{g}\cdot\text{ml}^{-1}$ (Figure 1 (b)).

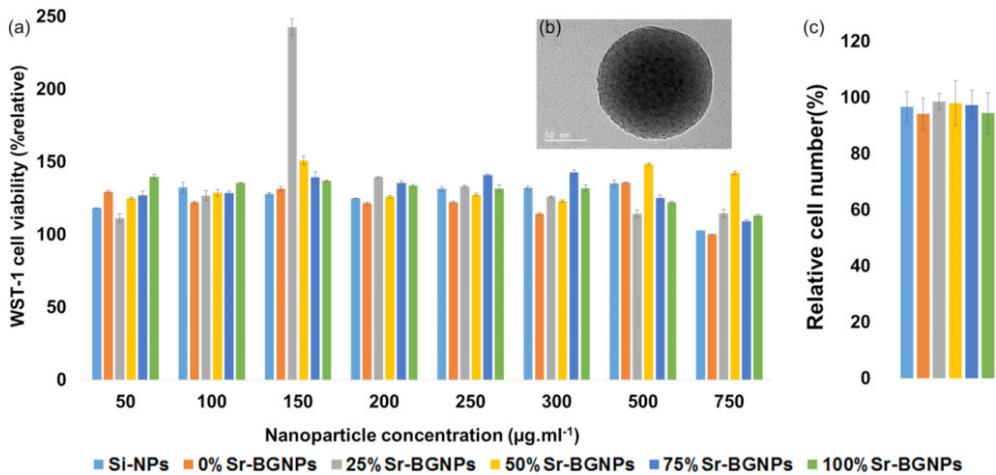


Figure 1: (a) Metabolic activity of macrophages after 24-hour incubation with different concentration of Sr-BGNPs, (b) TEM image of 75%Sr-BGNPs; scale bar=50 nm, and (c) total DNA of macrophages treated with different NPs at $200 \mu\text{g}\cdot\text{ml}^{-1}$

A DNA nick, an important indicator of cell apoptotic, was detected using a TUNEL assay. $200 \mu\text{g}\cdot\text{ml}^{-1}$ of Sr-BGNPs was used for the activation of macrophages for 24 hours. Apoptotic images confirm that there was no statistical difference in macrophage apoptosis between using the different percentages of Sr substitution on the mole basic (Figure 2). Taken together, these data indicate that Sr-BGNPs had no toxic effect on macrophages. To study the uptake of Sr-BGNPs in macrophages, cells were incubated for 24 hours with concentration of $200 \mu\text{g}\cdot\text{ml}^{-1}$ of 75% Sr-BGNPs. TEM images show an individual particle within a vesicle form bordered by a cell membrane (Figure 3a) and degraded particles inside the vesicle (Figure 3b). Confocal fluorescent images confirmed that 75% Sr-BGNPs internalized inside macrophages (Figure 4).

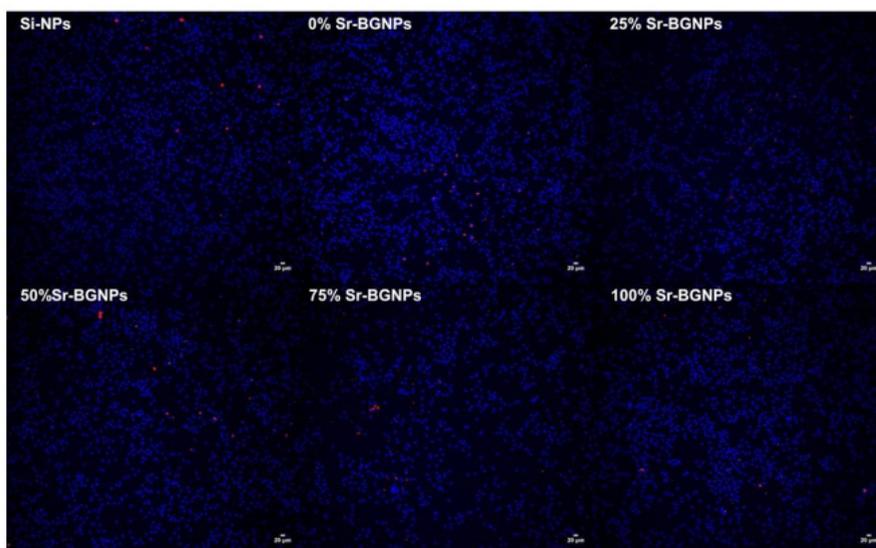


Figure 2: Untreated (control) and Sr-BGNPs treated of macrophages stained using the Invitrogen™ Molecular Probes™ Click-iT™ TUNEL Alexa Fluor™ 647 Imaging Assay; scale bar=20 μ m

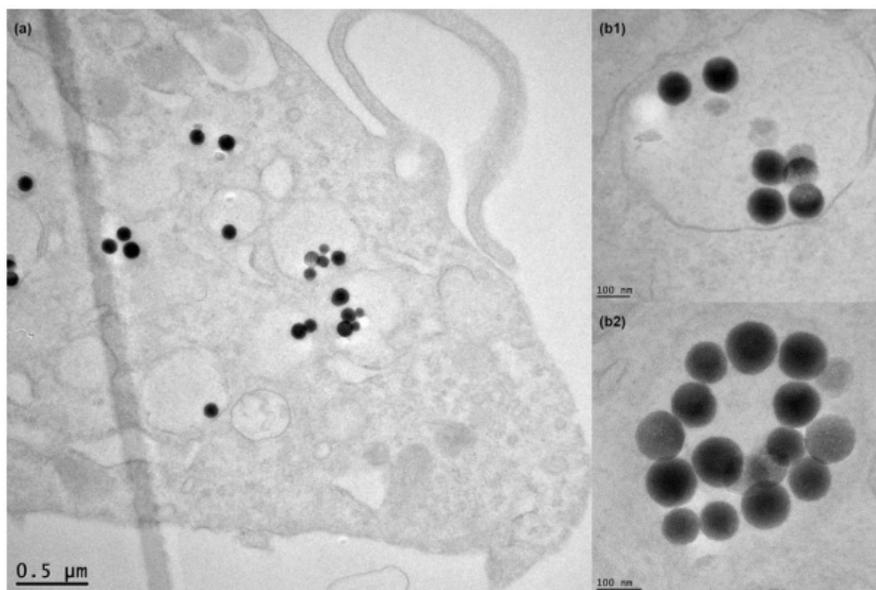


Figure 3: (a) TEM images of macrophages following incubation with 75% Sr-BGNPs for 24 hours; scale bar=0.5 μ m; (b) TEM images of degraded particles inside the vesicles; scale bar=100 nm.

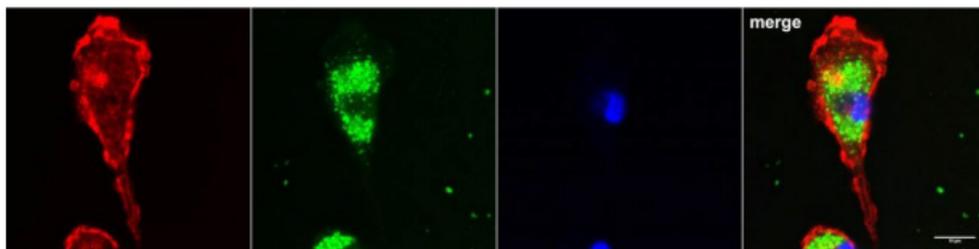


Figure 4: Confocal Microscopy Images of macrophages exposed to 75% Sr-BGNPs for 24 hours. Notes: red: phalloidin; green: FITC-75% Sr-BGNPs; and blue: DAPI; scale bar= 10 μm .

CONCLUSIONS AND/OR OUTLOOK

Sr-BGNPs are uptaken and localised by macrophages. The direct exposure to the different concentration of Sr-BGNPs ($50\text{-}750 \mu\text{g}\cdot\text{ml}^{-1}$) did not induced cell death and nuclear damage in macrophages. Based on this current work, Sr-BGNPs are potentially alternative materials for bone regeneration applications. Further studies are necessary to determine whether Sr-BGNPs are likely to be beneficial for promoting bone formation or be injurious immune responses.

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TREATMENT OF SKELETAL DISEASES WITH A NATURALLY DERIVED ANTIOXIDANT AND BISPHTHONATE EMBEDDED NANOPARTICLES

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Keywords: Bone, osteoblasts, chondrocytes, nanoparticles, gene expression

INTRODUCTION

Skeletal disorders are degenerative diseases causing progressive disability and are becoming more and more prevalent in our society. Bone is a special tissue able to support load and stress and is continuously renewed thanks to bone turnover mediated by the well coordinated activity of osteoblasts and osteoclasts. Many metabolic bone diseases are characterized by an imbalance of the activity of bone cells and bone turnover that could be estimated through the evaluation of laboratory data analysis. In addition to metabolic disorders, bone tissue can be impaired by genetic diseases such as osteogenesis imperfecta. In addition, Osteoarthritis (OA), the most prevalent musculoskeletal pathology, is predominantly characterized by the progressive degradation of articular cartilage due to an imbalance between anabolic and catabolic processes. Age-related changes that occur in articular are thought to represent a major risk factor for OA development. Therefore, skeletal diseases have been associated to defective differentiation pathways of progenitor stem cells (PSCs) to produce osteoblasts or chondrocytes. RUNX2 as well as SOX9 are transcription factors

responsible of commitment of PSCs in osteoblasts and chondrocytes respectively. In order to evaluate the possibility to affect and improve osteoblasts or chondrocytes commitment we assayed a naturally derived antioxidant and a bisphosphonate (clodronate) embedded nanoparticles in vivo (murine model) and in vitro (cell line) respectively. Antioxidant molecule was embedded into PLGA (poly lactic-co-glycolic acid) with the emulsion evaporation method. Bisphosphonate nanoparticles were prepared using chitosan and hyaluronic acid applying the ionotropic gelation method.

RESULTS AND DISCUSSION

We evaluated the effects of the antioxidant embedded nanoparticles treatment in mice by analysing the RUNX2 gene expression in bone tissue. As shown in Figure 1, Runx2 was overexpressed in treated mice respect to controls. In order to analyse the progression of the phenotype in committed mesenchymal cells we evaluated the gene expression of Sparc. We also assayed the ability to generate calcification nuclei by staining the CFU-Ob (Unit forming colony of osteoblasts) by alizarin red staining. Our data showed that treated mice expressed an increased Sparc gene (Figure2) and an higher number of CFU-Ob (Figure 3).

In order to evaluate the effects of bisphosphonate embedded nanoparticles on chondrocytes commitment, we treated human mesenchymal stem cell line and analysed the expression of SOX9 gene. As shown in figure 4 the treatment increased the expression of the transcription factor for chondrocytes commitment.

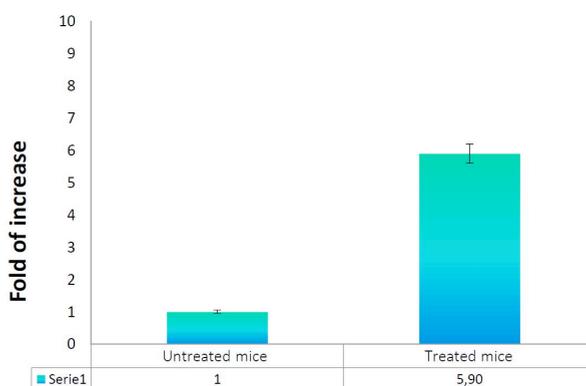


Figure 1: Runx2 expression levels in WT mice untreated and treated with anti-oxidant molecule nanoparticles

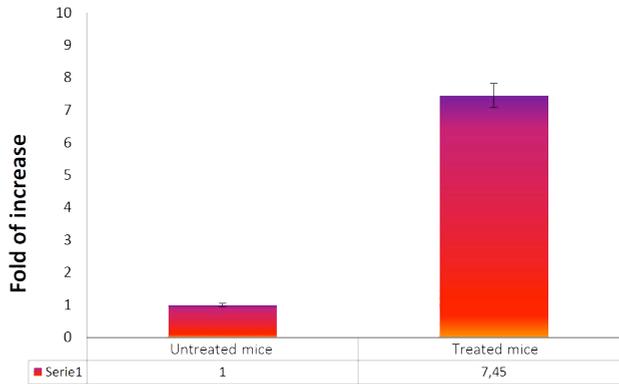


Figure 2: Sparc expression levels in WT mice untreated and treated with anti-oxidant molecule nanoparticles

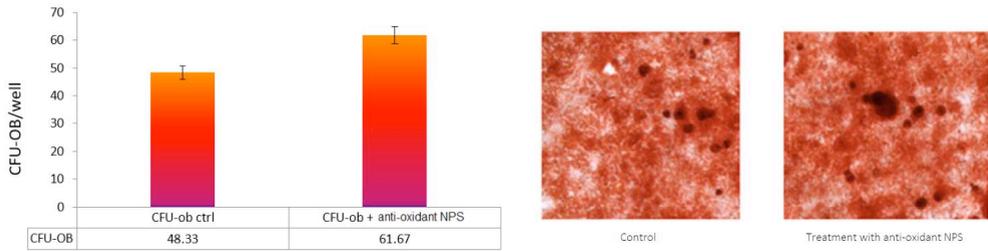


Figure 3: CFU-Ob analysis after anti-oxidant molecule nps treatment

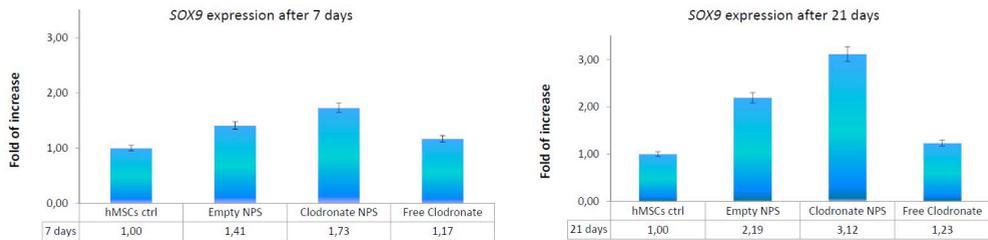


Figure 4: Analysis of SOX9 expression levels after 7 and 21 days nanoparticles treatment respectively

CONCLUSIONS AND/OR OUTLOOK

On the basis of our results, nanoparticles containing chemical formulation may represent a promising tool against bone cell degeneration. In conclusion, our findings support the possibility to use nanobiotechnologies to restore skeletal tissue in order to fight degenerative diseases and to improve life quality.

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ELECTROSPUN POLY (LACTIC ACID) FIBERS WITH SUSTAINED ANTIMICROBIAL ACTIVITY

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Key words: electrospinning, chlorhexidine particles, encapsulation, sustained release, antibacterial

INTRODUCTION

For chronic infection treatment, a local and sustained activity of antibacterial agents is the key to completely eliminate the bacteria. The conventional strategy of systemic administration of antibiotics will cause 'off target', adverse and side effects, and even resistance of bacteria. A lack of enough effect against bacteria may lead to second infection, and even result in the development of biofilms which are often more difficult to treat.[1] However, the requirement to maintain an adequate local drug concentration over a long period brings challenges to the design of drug carriers system. During the past decades, many systems (microparticles, nanogels and nanofibers) are developed for antibacterial agent delivery, and specially the nanofibers gain many interest because of their high surface area to volume ratio, porous and flexible properties and possibility of functionalization.[2] However, low drug loading capability and uncontrollable burst drug release limit their applications. Therefore, we came up the idea to directly incorporate chlorhexidine particles into the electrospinning poly (lactic acid) (PLA) fibers to improve the drug loading rate and pre-encapsulate the particles with layer-by-layer multilayers to achieve sustained release.[3]

RESULTS AND DISCUSSION

Chlorhexidine particles were fabricated by precipitation of chlorhexidine diacetate and

calcium chloride, and the homogeneous chlorhexidine spheres with average diameter (SD) of $17.15 \pm 1.99 \mu\text{m}$ showed an interconnected structure according to the SEM images. These particles had a high drug content and were used as templates for layer-by-layer self-assembly. After encapsulation, the needle-like surface morphology was covered by polyelectrolyte shells and relative smooth surface was displayed. Both the bare and encapsulated chlorhexidine particles were electrospun into PLA fibers and a bead-in-string structure was displayed since the diameter of chlorhexidine particles was much larger than the diameter (SD) of PLA fibers ($1.35 \pm 0.06 \mu\text{m}$). Chlorhexidine loading rate in the PLA fibers could be tuned by the amount of particles added, though with more chlorhexidine particles incorporated, diameter of fibers decreased slightly. Both types of fibers containing bare or encapsulated chlorhexidine particles showed a sustained release over 650 hours, but by pre-encapsulating the particles with layer-by-layer multilayers, the burst release at the beginning could be greatly reduced. The retention of chlorhexidine within the fibers was monitored by SEM as once the chlorhexidine released, collapse was observed and by confocal microscopy when the chlorhexidine particles were labelled. The incorporation of bare or encapsulated chlorhexidine particles into PLA fibers did not cause toxicity to healthy fibroblast according to the MTT assay or affect cells adhesion to fibers. In an agar diffusion assay, antibacterial activity against *E. coli* was observed for both type of fibers and the sustained effect was demonstrated in a series broth transfer assay.

CONCLUSIONS

We proposed a new strategy to load homogeneous drugs particles into electrospinning fibers and controlled release was achieved by layer-by-layer encapsulation of the drug particles. The produced PLA fibers containing bare and encapsulated chlorhexidine particles showed sustained activity and will have potential antibacterial applications in medicine and dentistry.

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EVALUATION OF PROANTHOCYANIDIN-CROSSLINKED ELECTROSPUN GELATIN NANOFIBERS FOR DRUG DELIVERING SYSTEM

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Keywords: Proanthocyanidin, Gelatin, Nanofibers, Electrospin

INTRODUCTION

The electrospun fibers form a porous scaffold which has a large surface area-to-volume ratio. Therefore, nanofibers are a promising material for many biomedical applications, such as wound dressings, drug delivery, and serving as a matrix template for tissue engineering [1,2].

Biodegradable materials are a more popular choice due to the elimination of a second surgery to remove the implanted scaffold [3]. Gelatin is a protein that contains high contents of glycine, proline, and hydroxyproline. Gelatin fiber is a biodegradable

polymer with excellent biocompatibility, plasticity, adhesiveness, cell adhesion and growth promotion, and low cost, making it ideal for use as a biomaterial. The use of a proper crosslinking agent to modulate the mechanical–chemical characteristics of gelatin is desirable for preventing toxicity and generating stable materials for biomedical applications [4,5]. The proanthocyanidin (PA) hydroxyl groups can interact with the gelatin carboxyl groups. Therefore, the PA compound appears to be a good candidate for such a role [6–9].

Proanthocyanidin (PA), a naturally occurring plant metabolite, has been adopted as an antioxidant, free-radical scavenger, and cardiovascular protector [10,11]. Proanthocyanidin (PA) is part of a group of polyphenolic compounds known as condensed tannins. They can stimulate normal skin fibroblast proliferation and increase the synthesis of an extracellular matrix, including collagen and fibronectin [12]. PA can be used to fix biologic tissues and biomaterials as well as stabilize biomaterials without cytotoxicity.

Conventional drug delivery systems such as nano or microspheres, liposomes, and hydrogels often initially have a burst drug-release problem. The drug release from electrospun fibers occurs via diffusion alone or diffusion and fiber degradation. The profile can be tailored by changing the content of the polymer mixture and the crosslinking time [13]. Our previous study has developed gelatin nanofibrous matrix that was prepared by electrospinning with glutaraldehyde crosslinking treatment. The results of *in vitro* biological evaluations demonstrated that the gelatin nanofibrous matrix fabricated by an electrospinning technique was found to enhance cell adhesion and proliferation [14]. This property makes electrospun fibers good candidates for topical/transdermal drug delivery [15,16].

In this study, the drug loading and release behavior of the Gel/PA blend fibers were examined by using magnesium ascorbyl phosphate (MAP) as a model drug. MAP, a derivative of ascorbic acid, is stable in water. MAP can be hydrolyzed to ascorbic acid by phosphatases in the liver or skin. Thus MAP exhibits vitamin C-reducing activity [17]. MAP can reduce melanin synthesis by inhibiting tyrosinase activity, which is why it has been widely prescribed as a skin-lightening and depigmenting agent [18,19]. We also evaluated the feasibility of adding PA to the gelatin electrospun nano-fibers. The improved mechanical properties and stable structure of the GEL/PA blend nanofibers were characterized by physical–chemical, biochemical, and cell culture studies. This gives electrospun fibers a potential application for controlled drug delivery. The high surface to volume ratio of electrospun fibers can enhance drug loading.

RESULTS AND DISCUSSION

Electrospun nanofibers are excellent candidates for various biomedical applications. We successfully fabricated proanthocyanidin-crosslinked gelatin electrospun nanofibers. Proanthocyanidin, a low cytotoxic collagen crosslinking reagent, increased the gelatin crosslinking percentage in the nanofibers from 53% to 64%, Figure 1. The addition of proanthocyanidin kept the nanofibers from swelling, and, thus, made the fibers more stable in the aqueous state. The compatibility and the release behavior of the drug in the nanofibers were examined using magnesium ascorbyl phosphate as the model drug. Proanthocyanidin also promoted drug loading and kept the drug release rate constant. These properties make the proanthocyanidin-crosslinked gelatin nanofibers an excellent material for drug delivery. In the cell culture study, L929 fibroblast cells had a significantly higher proliferation rate when cultured with the gelatin/proanthocyanidin blended nanofibers, Figure 2. This characteristic showed that proanthocyanidin-crosslinked gelatin electrospun nanofibers could potentially be employed as a wound healing material by increasing cell spreading and proliferation.

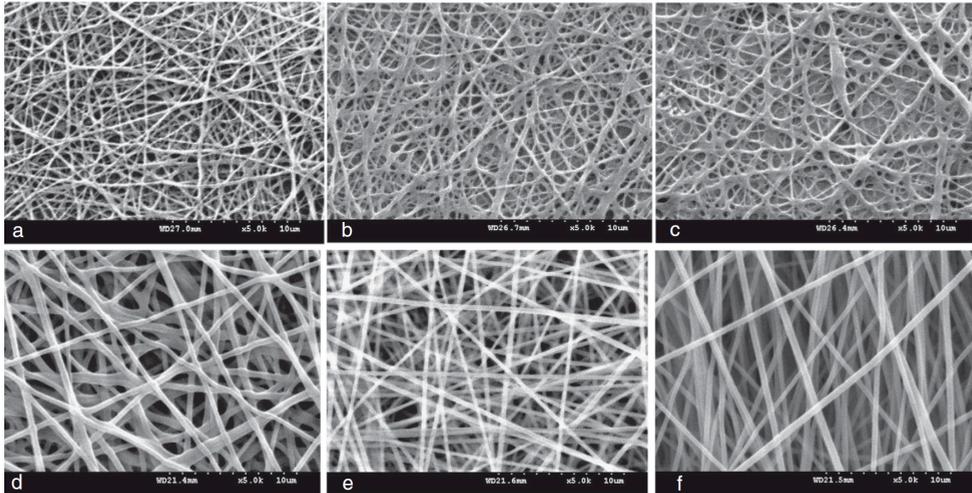


Figure 1: SEM photographs of the electrospun GEL and GEL/PA nanofibers crosslinked by 50 wt.% glutaraldehyde vapor for various periods of time.

(a) GEL, 15 min. (b) GEL, 45 min. (c) GEL, 90 min.

(d) GEL/PA, 15 min. (e) GEL/PA, 45 min. (f) GEL/PA, 90 min.

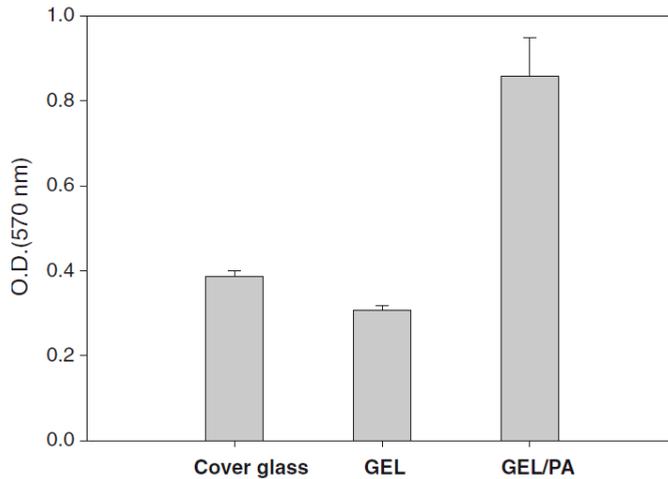


Figure 2: MTT assay of L-929 fibroblast cells that were cultured with glass, GEL, and GEL/PA nanofibers.

CONCLUSIONS AND/OR OUTLOOK

The addition of proanthocyanidin (PA) to the gelatin polymer solution can produce more stable, non-toxic and less swellable electrospun nanofibers. The GEL/PA nanofibers not only accelerate fibroblast cell proliferation, but also increase the drug loading efficiency. These properties make GEL/PA electrospun nanofibers an excellent candidate for wound dressing. Furthermore, based on the function of MAP in inhibiting melanogenesis, GEL/PA/MAP membrane might suppress melanin formation during wound healing process. This novel material might have great potential application in cosmetic products.

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MEMBRANE BIOREACTOR FOR THE LONG-TERM CO-CULTURE OF HUMAN HEPATOCYTES, ENDOTHELIAL AND MESENCHYMAL STEM CELLS

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Keywords: Membrane, Bioreactor, Liver, Human Hepatocytes, Mesenchymal Stem Cells, Co-culture

INTRODUCTION

Strategies to overcome in vitro dedifferentiation of hepatocytes preserving key features of the in vivo liver phenotype constitute a major goal in biomedical applications. Several types of co-culture systems have been performed to improve hepatocyte morphology and functions, by co-culturing them with different cells, including liver-derived cells, such as endothelial [1], sinusoidal, stellate and kupffer cells, or mesenchymal stem cells (MSCc) isolated from bone marrow, adipose tissue, umbilical cord and placenta.

In this study, we developed an organotypic membrane bioreactors by using primary human hepatocytes (hHeps) with human endothelial cells (hECs) and skin derived mesenchymal stem cells (hSSCs), which are MSCs isolated from human dermis [2]. hSSCs are less immunogenic, could be easily isolated and conveniently expanded, and therefore they could be a more promising and accessible cell source than MSCs from other origin, kupffer cells or hepatic stellate cells.

RESULTS AND DISCUSSION

An organotypic membrane bioreactor was developed by using hHeps, hECs and hSSCs, which were co-cultured between two flat-sheet gas-permeable membranes, which ensured the diffusion of O₂ and CO₂ and aqueous vapour between cells and external environment. This device allows a direct oxygenation of both cells adhered on the membrane and of the medium overlaying the cells, which is continuously supplied to the cells and well mixed. Optimal gas and nutrients exchanges were established, thus simulating the high perfusion and the sinusoidal organization of the in vivo hepatic microenvironment. The bioreactor was used in two different configurations: the first in which a direct co-culture was realized in a single compartment; the other one in which hHeps and hECs were separated from hSSCs by means of a porous semipermeable membrane that ensured the selective mass transfer of paracrine factors secreted by cells, avoiding the direct contact between hHeps/hECs and hSSCs. The organotypic membrane systems were maintained up to 19 days with high morpho-functional performance.

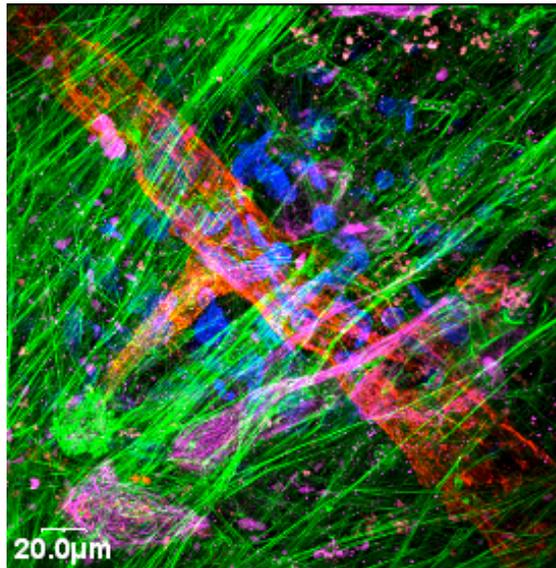


Figure 1: CLSM images of human hepatocytes, endothelial and mesenchymal stem cells after 19 days of culture in the oxygen permeable membrane bioreactor. Cells were stained for the cytoskeleton protein actin (green), the endothelial marker CD31 (red), the hepatobiliary marker CK19 (magenta) and counterstained for nuclei (blu).

CONCLUSIONS AND/OR OUTLOOK

The organotypic membrane bioreactors utilizing primary human hepatocytes in direct and connected co-culture systems with human endothelial and mesenchymal stem cells offered interesting opportunities for the design of bioartificial liver. Overall results demonstrated a strong influence on hepatocytes performance in terms of albumin secretion, urea synthesis and drug biotransformation functions.

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CYTOTOXICITY OF FUNCTIONALIZED GRAPHENE TO OSTEOBLAST-LIKE CELLS

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Keywords: graphene, biocompatibility, osteoblast, toxicity, nanoparticle

INTRODUCTION

Graphene is the thinnest material known to exist. It is also the strongest ever measured, being 100 times stronger than steel. During the last decade, graphene development has become one of the hottest topics in the fields of material science, physics, chemistry, and nanotechnology. One important research objective is to elucidate the interactions between graphene and bioorganisms.^{1, 2} In recent advances, graphene is being used in diagnostic tools and chips.³ Graphene is a suitable material for nanocomposites due to its great stiffness and strength.⁴ Polymer/graphene composites possess improved mechanical properties.⁵ Since graphene-composites can create a port of entry for carbon nanoparticles into the body, it is imperative to learn more about the cytotoxicity of graphene. Great care must be taken to ensure that this nanomaterial's toxicity is well defined and understood. The interaction between cells and artificial substrates is of particular interest. In this study we used aqueous emulsifier-free FG dispersions (FG-D), derived from thermally reduced graphite oxide (600 m²/g), as a model system to examine the effect of FG on in-vitro cytotoxicity. The cytocompatibility and the proliferation of different bone markers are assessed using MG 63 cells.

RESULTS AND DISCUSSION

We report the synthesis, dispersion, and cytotoxicity of FG (600 m²/g), prepared by thermally reducing graphite oxide. As a function of the dispersion process and dispersion medium, it was possible to vary size and shape of FG without changing functionality. While suspension of FG in culture media afforded large micron-sized FG-assemblies (FG-S), able FG nanosheet dispersions (FG-D) were obtained upon high pressure homogenization in water. It was possible to add FG-D to culture media without encountering FG agglomeration. Determination of cell viability, cell proliferation, cell shape analysis including detection of plasma membrane integrity and osteogenic cell differentiation show a good cytocompatibility of FG, independent of its size, shape, functionality, and surface area. The proliferation of different bone markers is also identical. There is no internalization of FG in osteoblast like cells up to 14 days. FG has a high adsorption capacity of proteins and cells, owing to the presence of functional groups. A high FG concentration of 100 µg/mL can lead to artefacts in biological assays and other investigation methods. To the best of our knowledge, this is the first study that examines the behaviour of osteoblast like cells over a prolonged period of exposure to FG.

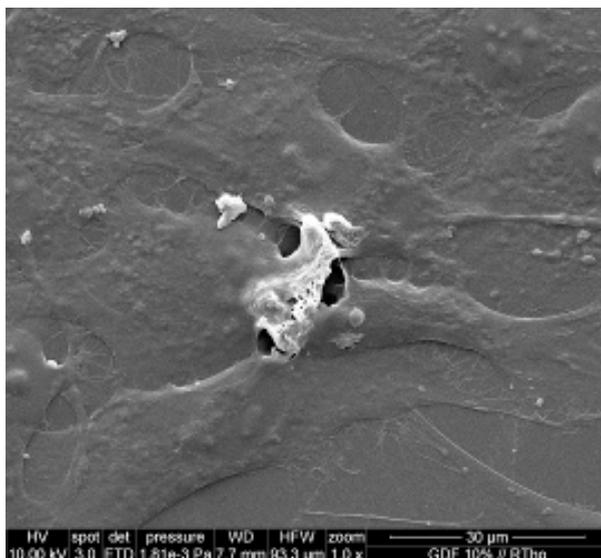


Figure 1: ESEM image after treatment of cells with FG sheets.

CONCLUSIONS AND/OR OUTLOOK

Our studies have demonstrated that a high specific surface is resulting in a strong interactions of FG with the plasma membrane of cells. This process is not combined with cytotoxic effects up to 10 µg/mL. A concentration of 100 µg/mL can lead to artefacts in biological assays and other investigation methods concerning toxicity because of the very high surface area of TRGO. High protein adsorption followed by strong cell adsorption. This provides strong evidence that FG, independent of its size and shape, is highly cytocompatible below concentration of 100 µg/ml.

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POLY(VINYLLIDENE DIFLUORIDE-TRIFLUOROETHYLENE) SMART PIEZOELECTRIC COMPOSITE FILMS WITH BORON NITRIDE NANOTUBES FOR BIOMEDICAL APPLICATIONS

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Keywords: Piezoelectricity, BNNTs, P(VDF-TrFE), cell culture.

INTRODUCTION

Wireless electrical stimulation of biological environments is an approach that is attracting increasing interest in the biomedical research aiming at the regeneration of electrically responsive tissues and at the recovery of their function after trauma or disease. Piezoelectric materials respond to mechanical stimulations provided for instance under the form of ultrasounds (US) or vibrations by undergoing deformation and subsequent polarization. Since 2010, our group explores this effect in boron nitride nanotubes (BNNTs) and other piezoelectric nanoceramics in order to trigger and properly direct biological responses [1-3]. Contemporarily, an increasing number of evidences in the literature have started to emerge supporting safety and effectiveness of piezomaterials in stimulating cells in vitro [4,5].

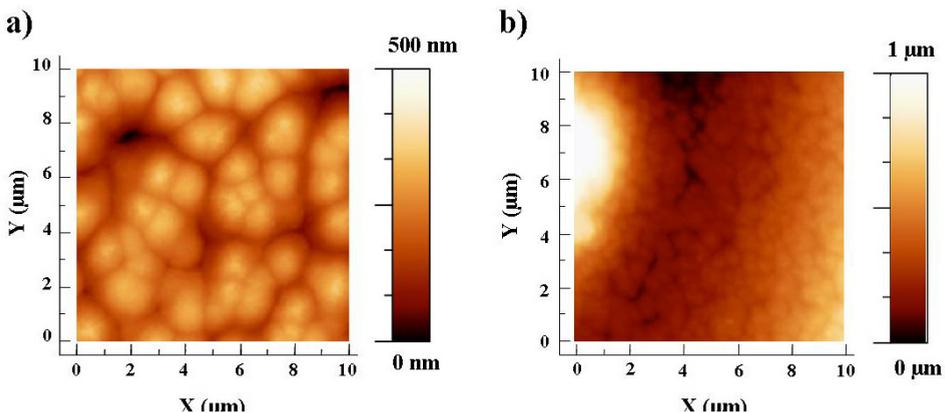
RESULTS AND DISCUSSION

In this work, piezocomposite films of poly(vinylidene difluoride-trifluoroethylene) (P(VDF-TrFE), 70-30 mol % copolymer) and BNNTs were prepared to be used as substrates for the culture and electrical stimulation of SH-SY5Y cells. The preparation of composite films aimed at merging the excellent mechanical properties of the polymeric piezoelectric component to the superior piezoelectric response of the ceramic component.

To pursue our aim, we mixed P(VDF-TrFE) and BNNTs at a 90:10% (w/w) ratio in methylethylketone (MEK). Composite films were prepared by mixture ultrasonication for 10 min at 8 W, casting of 800 μl on 9 cm^2 Ibidi film, and annealing at 40°C for 4 h. To increase surface hydrophilicity and thus cell adhesion, films were exposed to O₂ plasma at 60 W for 120 sec. Prior to cell culture, films were extensively characterized in terms of surface and bulk properties, by using for instance scanning electron/ transmission microscopy and atomic/piezoresponse force microscopy.

SH-SY5Y cells were seeded at a density of 10,000/ cm^2 and differentiated with routine culture media. As control substrates, cyclic olefin copolymer Ibidi film and P(VDF-TrFE) (20% in MEK) were considered. After 5 days of differentiation, US stimulation was performed by applying the following parameters with a KTAC 4000 Sonoporator: 2 W, 100% duty cycle, 100 Hz burst rate, 5 sec. Transcriptional levels of c-Fos, an early marker of neuron electrical activity, were assessed on cultures at 10 min from US exposure with quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR).

BNNTs were found to be homogeneously dispersed in the films and over the surface (compare Figure 1a and 1b). c-Fos transcriptional levels were strongly up-regulated by cell growth on BNNT films and exposure to US compared to both control substrates (Figure 1c).



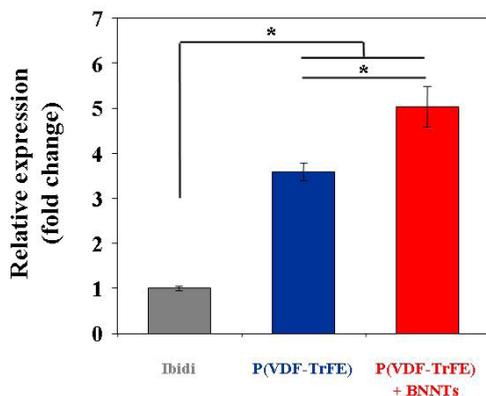


Figure 1: Atomic force microscopy maps of plain P(VDF-TrFE) films (a) and P(VDF-TrFE)/BNNT composite films (b). qRT-PCR analysis of c-Fos (Ldha as reference gene). * $p < 0.01$.

CONCLUSIONS AND/OR OUTLOOK

Although preliminary, our result represents a first evidence of piezoelectric transduction mediated by P(VDF-TrFE)/BNNT composite films, that is able to trigger SH-SY5Y response, even higher than that achievable by plain P(VDF-TrFE) films, and encourages further exploration of P(VDF-TrFE)/BNNT composite films potentialities for the electrical stimulation of neurons.

ACKNOWLEDGEMENTS

Francesca Pignatelli and Sergio Marras (Istituto Italiano di Tecnologia) are gratefully acknowledged for technical assistance in film characterization. This research has been partially supported by the Italian Ministry of Health, Grant Number RF-2011-02350464.

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MESENCHYMAL STEM CELLS COATED WITH SYNTHETIC BONE-TARGETED POLYMER AS A NEW APPROACH FOR MANAGING OF OSTEOPOROTIC BONE FRACTURE REGENERATION

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Keywords: osteoporosis, mesenchymal stem cells, ATRP synthesis, targeted cell delivery

INTRODUCTION

Osteoporosis is characterized by compromised bone strength that predisposes patients to an increased risk of fracture. Osteoporotic patients differ from normal subjects in bone mineral composition, bone mineral content, and crystallinity. The reduction of bone mineral density or osteopenia is caused by increased rate of resorption during bone turnover, age-related reduction in osteoblasts progenitor cells and reduced calcium absorption. Much effort has been expended on improving therapies that are expected to preserve bone mass and thus decrease fracture risk. Yet, less importance has been given to investigating fracture healing in osteoporosis compared to studies focused on preventing osteoporotic fractures. There are a number of reports indicating that the transplantation of bone marrow-derived MSCs promote bone formation at sites of bone defects. However, only few of them are addressed to osteoporotic fractures, and more research is needed.

In the present study we have tested local transplantation of mesenchymal stem cells

in order to stimulate the process of osteoporotic bone fracture regeneration. To ensure the bone targeting moiety to MSCs, the cells were coated with synthetic osteophilic polymer synthesized using atom transfer radical polymerization (ATRP). The primary active sites of the polymer are bisphosphonate functional groups that target hydroxyapatite molecules (HA) on the bone surface. NHS groups on the other end of the molecule allow polymer to bind to the cell surface components. Coating of MSCs with the polymer allowed the cells to bind specifically to HA component of bone and localize the cellular repair functions to areas of injured bone. The inclusion of fluorescein in the polymer allows visualization of the polymer binding to both bone fragments and to the cells.

RESULTS AND DISCUSSION

Previous *in vitro* studies showed that polymer can be stably attached to cell surface for at least 4 hours and to bone fragments *in vitro* for at least 3 hours, confirming the bone targeting potential of the polymer. The polymer was not shown to be cytotoxic by cell viability assay (Figure 1). and did not affect further differentiation of MSCs into osteocytes (Figure 2).

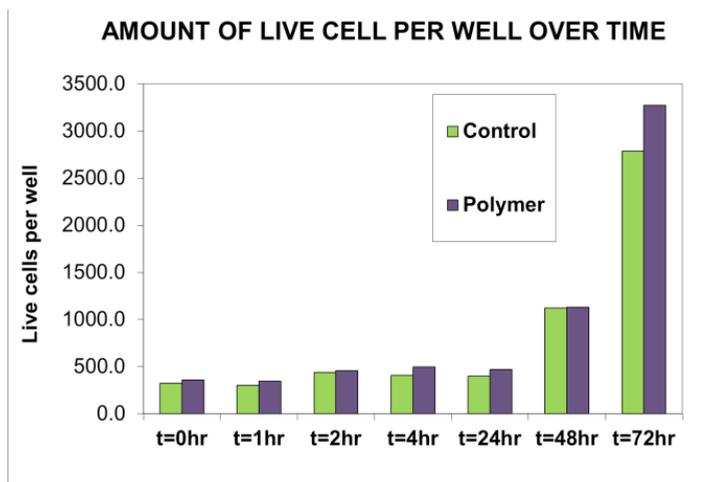


Figure 1: MSCs ($1.0 \pm 1 \times 10^6/\text{ml}$) were incubated with 1 mg/ml of polymer for 10 min, washed 3 times and plated on 96-well microplate. At certain time point (0hr, 1hr, 2hr, 4hr, 24hr, 48hr, 72hr – stored in incubator at 37°C , $5\%\text{CO}_2$) the reagent (Luminescent Assay) was added and the luminescent signal was read using Synergy Hybrid Reader (Biotek, USA).

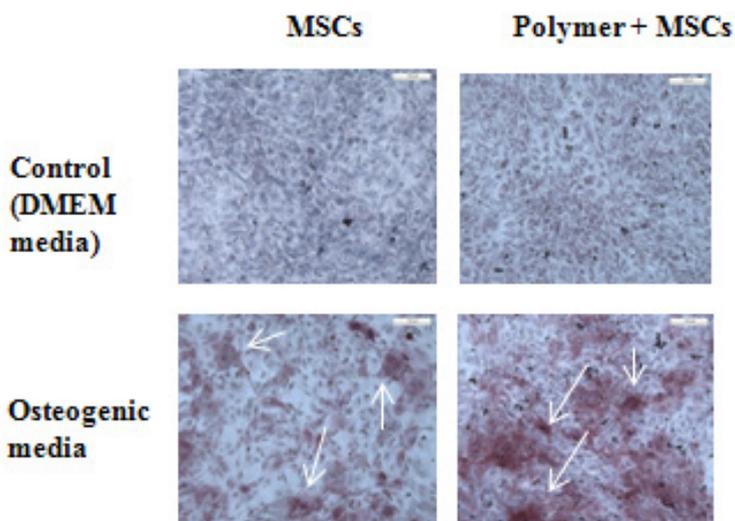


Figure 2: Effect of the polymer on differentiation of MSCs down the osteogenic lineage. MSCs ($1.0 \pm 1 \times 10^6/\text{ml}$) were incubated with 1 mg/ml of polymer for 10 min, washed 3 times, plated on 24-well microplate, and stored in incubator at 37°C , $5\% \text{CO}_2$. Regular media was substituted by osteogenic after 12 hours. Control MSCs were cultured in regular media. Plates were assessed on ALP activity on day 12. Regions with high ALP activity (dark pink) indicated the process of osteogenic differentiation (shown with arrows) and by visual assessment are similar between polymer modified MSCs and control group.

Osteoporosis-like condition in rats was experimentally induced by bilateral ovariectomy and confirmed via measuring bone density and histological assessment. Ulna fracture model was performed in 4 groups (5 animals each) and each group received different solution (Polymer in PBS, MSCs in PBS, MSCs+Polymer in PBS). Group I served as a control (PBS only). Injections were administered locally at the site of the fracture every week during 1 month. Bone density was measured locally at the zone of fracture right after operation and 4 weeks later with Micro-CT tomography. Data are presented as ratio of final bone density (4 weeks after fracture) to initial (just post-surgery). Our data demonstrated significantly increased bone density in fracture zone in group D (34%) compared to sham control (figure 3). Histological assessment showed formation of the young bone tissue from immature cells at fracture zone (Figure 4). Thus, our new method based on targeted delivery of mesenchymal stem cells coated with polymer modified with bisphosphonate side chains has a potential for improving clinical outcomes for fragility fracture patients and requires further investigations.

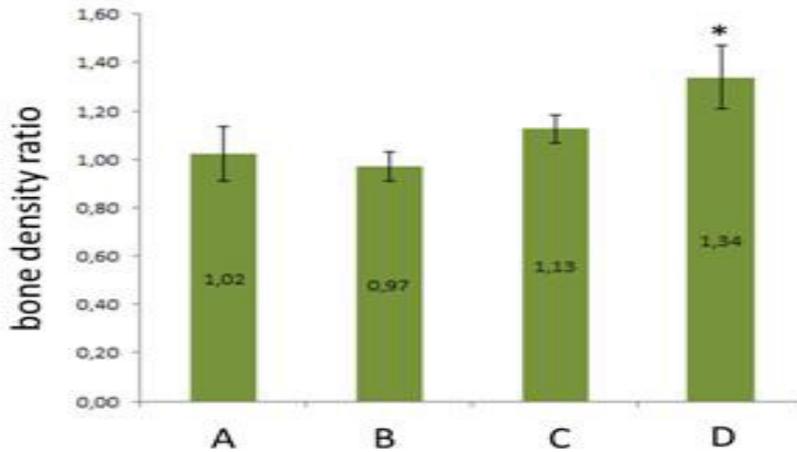


Figure 3: Data is presented as proportion of final bone density after 4 weeks of injection compared to the initial (bone fracture). P value = 0.006 compared to the control group, total number of animals n=20, 5 animals per group). Group A (5 animals) – sham control (only PBS); group B (5 animals) – 200 μ l polymer in PBS in concentration 1 mg/ml; group C (5 animals) - 200 μ l PBS containing MSCs (1×10^6 cells/ml); group D (5 animals) - 200 μ l PBS containing MSCs modified with polymer (1×10^6 cells/ml).

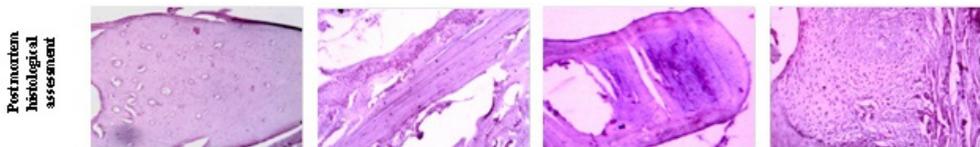


Figure 4: Histological assessment of cross-section of cortical bone: A - Fracture zone : degenerative changes in the cells of bone tissue with signs of vacuolization and nuclear deformation H&A stain, 200X; B - Fracture zone: the formation of new trabecular bone - fine crossbar with signs of weak mineralization, H&A stain, 200X; C - Fracture zone: irregular mineralization of bone tissue with areas of calcification spotted, H&A stain, 200X; D - Fracture zone: the young bone tissue formed from immature cells., H&A stain, 200X.

A TISSUE ENGINEERING STRATEGY USING PLACENTAL MESENCHYMAL STEM CELLS HOSTED ON RKKP GLASS-CERAMIC FOR BONE REGENERATIVE MEDICINE APPLICATIONS

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Keywords: human amniotic mesenchymal stromal cells (hAMSCs), regenerative medicine, glass-ceramics, RKKP films, pulsed laser deposition.

INTRODUCTION

In tissue engineering protocols, the survival of transplanted stem cells is a limiting factor that could be overcome using a cell delivery matrix able to support cell proliferation and differentiation. With this aim, using human amniotic mesenchymal stromal stem cells (hAMSCs) from placenta, the cell-friendly and biocompatible behaviour of RKKP glass-ceramic coated Titanium (Ti) surface was studied. The glass-ceramic material was prepared by sol-gel synthesis procedure and then deposited onto the Ti surface by Pulsed Laser Deposition method.

RESULTS AND DISCUSSION

The cell metabolic activity and proliferation rate in the hAMSCs seeded on the RKKP glass-ceramic surface did not differ from those on the Petri dish. The cytoskeletal actin organization, the cell cycle study and the immunophenotypical characterization, revealed no significant differences in their cycle phase distribution, mesenchymal CD29 and CD73 markers' expression, and cell morphology when compared to the cells grown on the Petri dish. The healthiness of hAMSCs was also analysed studying the mRNA expressions of MSC key genes and the osteogenic commitment capability, which results unchanged in both substrates.

Figure 1: Placental Mesenchymal Stem Cells hosted on RKKP glass-ceramic

CONCLUSIONS

The combination of the hAMSCs' properties together with the bioactive characteristics of RKKP glass-ceramics were investigated and the results obtained indicate its possible use as a new potential tool for bone tissue engineering and regenerative medicine applications.

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HALLOYSITE AND CHITOSAN OLIGOSACCHARIDE NANOCOMPOSITE FOR WOUND HEALING

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Keywords: Halloysite, chitosaoligosaccharide, nanocomposites, wound healing

INTRODUCTION

Halloysite is a natural clay belonging to the kaolin group and based on nanotubes (one dimensional nanoparticles) with hollow nanotubular structure having an inner diameter of 10–30 nm and an outer diameter of 50–70 nm and a length in the range of 0.5–1.5 μm . Halloysite is two-layered aluminosilicate ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot n\text{H}_2\text{O}$), with aluminol (Al-OH) groups in the internal surface and Si-O groups on the external surface. This chemical difference results in a negatively charged outer surface and positively charged inner lumen surface at the relatively wide pH range, which is beneficial for further functionalization^{1, 2}.

Due to the inexpensive availability, high specific surface area, large aspect ratio, and good biocompatibility, Halloysite nanotubes (HNT) are attractive nanomaterials in the vast range of biological and nonbiological applications including nanoscale container of biologically active molecules.

Chitosan oligosaccharides (COS) are homo- or heterooligomers of *N*-acetylglucosamine and *D*-glucosamine, prepared starting from chitin or chitosan and by using enzymatic conversions. It is reported in literature that COS accelerate wound healing by enhancing the functions of inflammatory and repairing cells and are characterized by

antibacterial and antifungal activities.

Given these premises, the aim of the work was the development of nanocomposites based on halloysite nanotubes and COS, intended for the treatment of chronic skin lesions as a powder for cutaneous application, to enhance wound healing and to prevent wound infections.

RESULTS AND DISCUSSION

Figure 1 reports % viability of fibroblasts after 3 and 24 h of contact time with HL (300 mg/ml concentration), HL/COS (nanocomposite with HL at 300 mg/ml concentration and COS at 0.2 mg/ml concentration), COS (0.2 mg/ml concentration). After 3 h contact time, HL showed a decrease in cell viability. All the other samples showed viability comparable to that of growth medium (positive control, 100% viability). After 24 h all the samples were characterized by viability close to 100%.

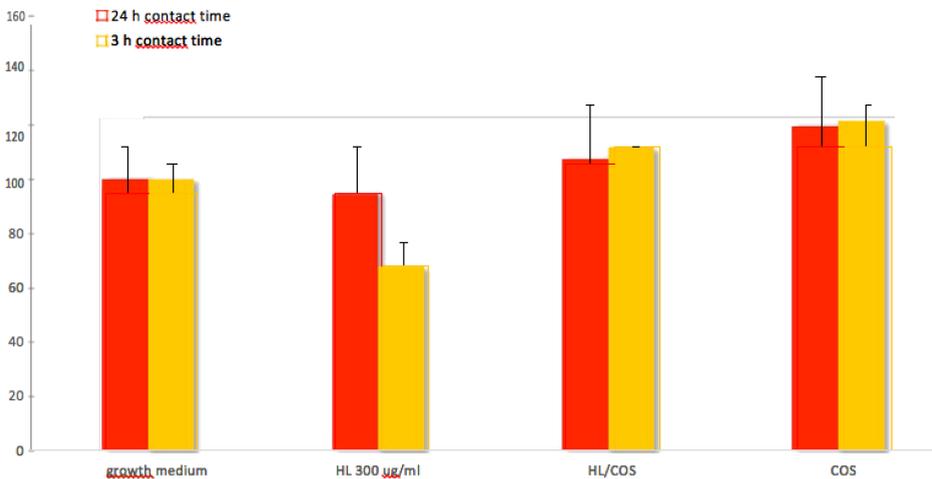


Figure 1: % viability of fibroblasts after 3 and 24 h of contact time with HL, HL/COS (nanocomposite), COS (mean values \pm sd; n=8)

Figure 2 reports the microphotographs of fibroblasts after different contact times (from 0 to 72 h) with HL (300 mg/ml concentration), HL/COS (nanocomposite with HL at 300 mg/ml concentration and COS at 0.2 mg/ml concentration), COS (0.2 mg/ml concentration) in an in vitro wound healing test. HL/COS nanocomposite and HL and COS alone demonstrated to enhance fibroblast motility, showing fibroblast proliferation and migration similar to that of standard growth medium (containing growth factors), leading to gap closure within 48 h of contact time.

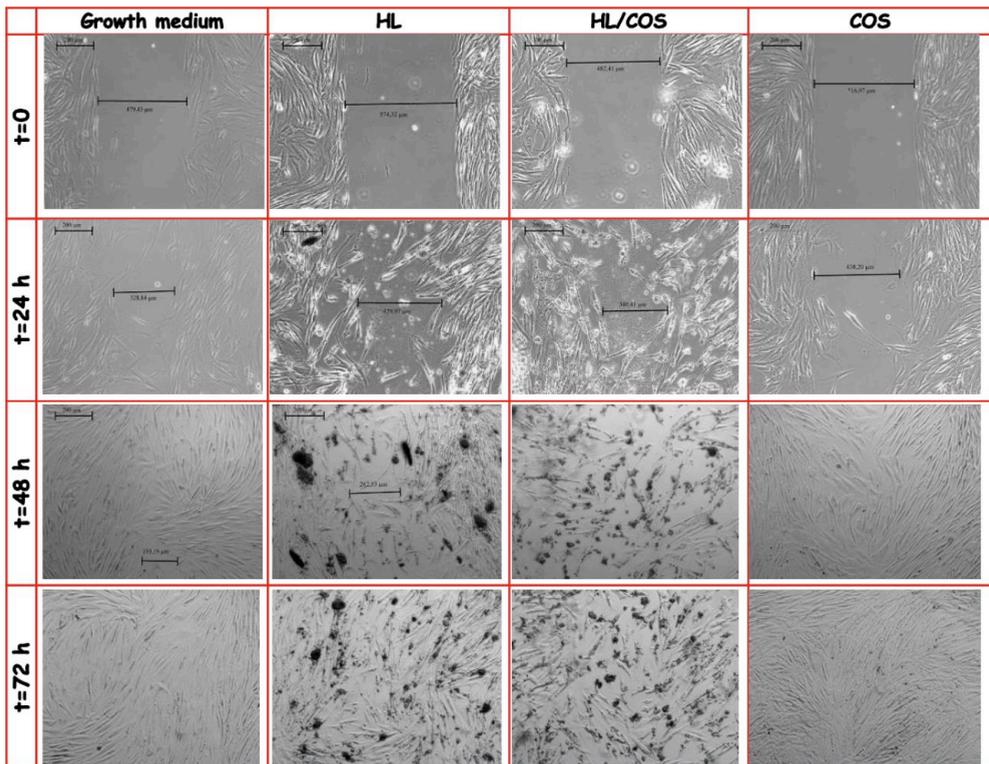


Figure 2: microphotographs of fibroblasts in contact with with HL, HL/COS (nanocomposite), COS in in vitro wound healing test

CONCLUSIONS

Nanocomposite demonstrated to be biocompatible towards fibroblasts in in vitro tests. Moreover it has been demonstrated to enhance fibroblast motility and proliferation in an in vitro wound healing test, as standard growth medium (containing

growth factors), with gap closure within 48 h of contact time. Even if microbiological evaluation and in vivo evaluation (murine model) are still in progress, this system seems promising to enhance skin wound healing.

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INSULIN NANOGELS: A NEW STRATEGY FOR THE TREATMENT OF ALZHEIMER'S DISEASE

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A growing body of evidence shown that Insulin, Insulin Receptor (IR) and IR signaling are involved in brain cognitive functions and their dysfunction is implicated in Alzheimer's disease (AD) degeneration. Thus, administration of insulin could be a strategy for AD treatment. For this aim we designed, synthesized and characterized a nanogel system (NG) to deliver insulin to the brain, as a tool for the development of a new therapy for AD. A carboxyl-functionalized poly(N-vinyl pyrrolidone) nanogel system produced by ionizing radiation was chosen as substrate for the covalent attachment of insulin or fluorescent molecules relevant for its characterization. Biocompatibility of the naked carrier was demonstrated by absence of cytotoxicity, oxidative stress and mitochondrial dysfunction. Hemocompatibility was demonstrated by hemolysis, coagulation time, leukocyte proliferation and inflammatory response tests. By immunofluorescence measurements we demonstrated that insulin conjugated to the NG (NG-In) is protected by protease degradation and is able to bind and activate insulin receptor bringing to trigger the insulin signaling via AKT activation. Moreover, to provide consistent evidence on the functionality of the conjugated insulin on the glucose levels, the effect of NG-In was tested in mice demonstrating that the plasma glucose levels was reduced. Neuroprotection of NG-In against dysfunction induced by amyloid β , a peptide mainly involved in AD, was verified. Finally, the potential of NG-In to be efficiently transported across the Blood Brain Barrier was demonstrated by using an *in vitro* system. All together these results indicated that the synthesized NG-In

was a suitable vehicle system for insulin delivery in biomedicine and a very promising tool to develop new therapies for neurodegenerative diseases. The research was supported by MIUR, Flagship Project NanoMAX.

NEURONAL MEMBRANE PLATFORMS AS TOOLS FOR ALZHEIMER'S DISEASE TREATMENT

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Keywords: Membrane bioreactor, neuronal cells, Alzheimer's disease, microtube array membrane

INTRODUCTION

Among the different devices that have been successfully applied in neuro-biotechnological field, membrane systems are promising approaches for culturing neuronal cells, offering a homogeneous environment in which cells are well supplied and the removal of catabolites is ensured. Within this scenario, our strategy was to develop high performing neuronal membrane bioreactors as a platform for the in vitro reconstruction of neuronal network with defined functional, geometric and neuroanatomical features [1]. The ideal microenvironment recreated in these systems, which promoted neuronal maturation and axonal sprouting and elongation, allowed their use as in vitro platforms for studying neuroprotection in Alzheimer disease. These devices have been also used to test the capacity of neuronal cells to react to topographical stimuli thus guiding their orientation.

RESULTS AND DISCUSSION

The bioreactors configuration ensured an optimal cell perfusion granting neurons an equal distribution of nutrients and oxygen and the constant removal of waste products. Within the bioreactors, neurons have a wide membrane surface for cell adhesion; indeed cells cover an extensive membrane area as a proof of a good interaction

at the cell-biomaterial interface. The satisfactory cell-material synergism favoured an adequate neuronal maturation and differentiation, which leads to the formation of a highly branched neuronal network with a three-dimensional cell arrangement, pointing out the persistence cell integrity as evidenced in figure 1. The acquisition and maintenance of a neuronal phenotype onset was confirmed by the localization, through laser scanning confocal microscopy images, of specific neuronal markers as GAP-43, β III tubulin (Tuj1) and synaptophysin. The ideal microenvironment recreated within the poly-acrylonitrile (PAN) hollow fiber bioreactor suggested its use as in vitro model system to study the anti-amyloidogenic effect of the carotenoid crocin [2]. It was demonstrated that crocin protects neurons from Ab induced toxicity both preventing ROS generation and the apoptotic cascade, thus arresting cell death. The set of data demonstrated that crocin exerts its action inhibiting the formation of Ab fibrils and thus neutralizing its toxicity. The use of poly-L-lactic acid (PLA) microtube array membrane bioreactor besides promoting the maintenance of neuronal specific features guides neurons towards the definite pathway offered by the highly aligned membrane. The membrane disposition directed the neuronal process orientation according to the microtube's one. Indeed the neuronal guidance offered by the PLLA microtube array membrane bioreactor suggests that it can be used as bridging device able to enhance nerve repair and/or regeneration.

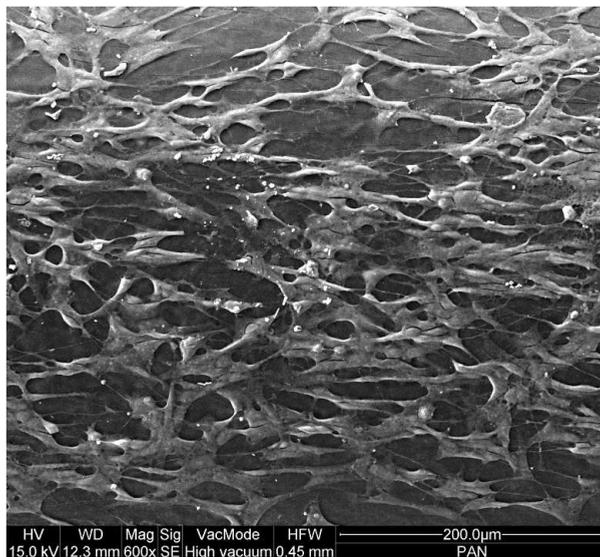


Figure 1: SEM micrographs of neuronal cells after 14 days of culture in the PAN HF membrane bioreactor.

CONCLUSIONS

These devices offer a broad range of application in the field of tissue engineering and regenerative medicine. Neuronal membrane bioreactors allow to perform cutting edge investigations in neurobiological field. They represent both valuable in vitro platforms for the investigation of neurodegenerative diseases with a strong impact on modern society and innovative devices to be used in restoring peripheral nerve damage by boosting its regeneration.

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A THERMO-SENSITIVE HYDROGEL COMPOSED BY OXIDIZED METHYLCELLULOSE AND ADIPIC ACID DIHYDRAZIDE AS AN ANTI-OXIDANT CARRIER ON TRAUMATIC BRAIN INJURY

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Keywords: Traumatic brain injury, free radical, ascorbic acid, oxi-methylcellulose, anti-oxidant carrier.

INTRODUCTION

Traumatic brain injury (TBI) is an important global public health problem. It is a major cause of disable and traumatic death [1, 2]. TBI often occurs when a sudden trauma causes damage to the brain by external force. Secondary brain injury then occurs by the free radical damage. Free radicals were major cause to the failure of TBI treatments afterwards. Therefore, it became an important procedure that eliminating the possibility of the brain tissue suffering from oxidation pressure by scavenging the free radicals at injury site. Recently, the reports indicated that low molecular weight antioxidants (LMWAs), such as Vitamin C and Vitamin E, constitute the component of the anti-oxidant defense mechanism [3]. However, due to taking long time to circulate to injury site, the antioxidant activity could be lost and lead to the poor outcome of therapeutic effect. Therefore, a carrier for local releasing of anti-oxidant into injury site is necessary. In this study, an anti-oxidant carrier we developed, which

is composed of oxidized methylcellulose (oxi-MC) and adipic acid dihydrazide (ADH), could continuously release for TBI treatment.

RESULTS AND DISCUSSION

The aldehyde group in the backbone of methylcellulose was created by reacting with sodium meta-periodate, and the oxidation rate of oxi-MC created were 21% approximately. The oxi-MC/ADH hydrogels ensures an excellent clinical expediency with a gelation time within 5 minutes on body temperature. The biocompatibility evaluation was confirmed that there are no significant cytotoxic, and biodegradable of oxi-MC/ADH hydrogel. We also evaluated the release profile of ascorbic acid-incorporated oxi-MC/ADH hydrogel, the initial burst was discovered within 24 hours, and released continuously through a week. The release profile is suitable for treating secondary TBI. The gene expression profiles suggested that the neuro-protective of ascorbic acid down-regulated oxidative stress, inflammatory, and apoptosis related genes on H_2O_2 inducing cells. The result of CM-H₂DCFDA fluorescence staining also indicated that this hydrogel could lead cell enduring oxidative stress. The TBI animal model was created on male Sprague–Dawley rats by a cortical impact device. Ascorbic acid incorporated oxi-MC/ADH hydrogel was injected into injury site for treatment. Neurological function was then evaluated by using modified Neurological Severity Score (mNSS). This results indicated that the recovery has seen significant improvements between hydrogel treated and untreated group after 3 weeks. The results of clinical chemistry in serum and hematology assay indicated that the hydrogel would not trigger cytotoxic effect after being implanted for a month.

CONCLUSIONS AND/OR OUTLOOK

In the clinical practice for traumatic brain injury, ideal treatment method has not been discovered. In this study, we established the synthesis and characterization of a suitable anti-oxidant carrier composed of methylcellulose. The hydrogel displays several benefits for application of TBI treatment. These advantages include quick gelation on body temperature, biodegradable, and controlled release of anti-oxidant. The hydrogel also exhibited great biocompatibility with rat neuro-blastoma cell. In the animal study, the hydrogel with anti-oxidant could improve the TBI rats recovered. Summarizing the results, we suggested the oxi-MC/ADH hydrogel incorporating ascorbic acid as a potential anti-oxidant carrier for TBI treatment.

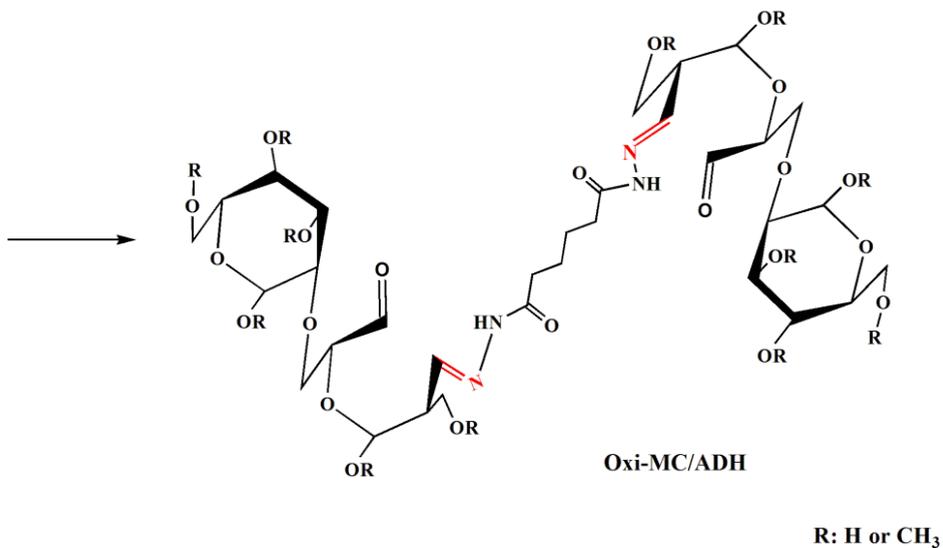
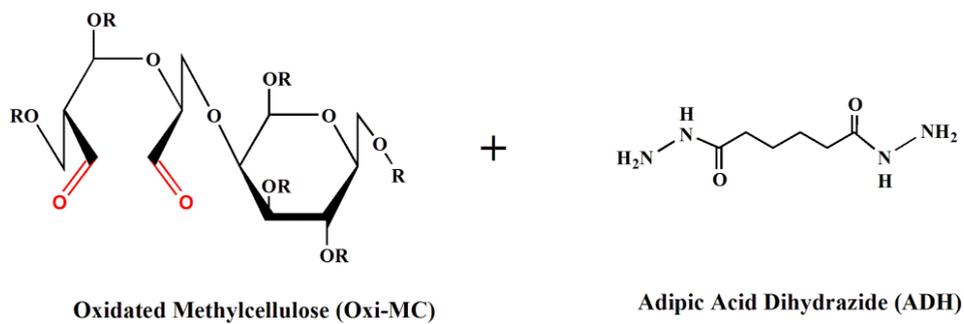
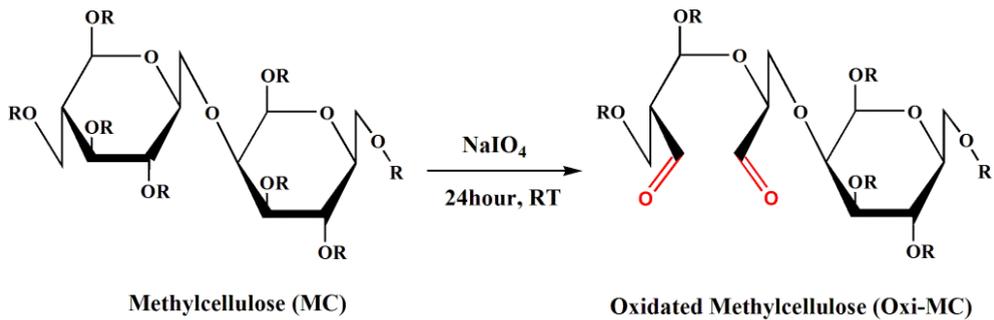


Figure 1: The chemical cross-linking mechanism of oxi-MC/ADH hydrogel.

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MULTILAYER HYDROGEL SYSTEMS FOR THERAPEUTIC ANGIOGENESIS

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Keywords: hydrogels, drug delivery system, viscoelastic properties

INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of mortality in many European countries¹. This comprises ischemia which is characterized by a suppression of blood supply to a tissue. New strategies such as the use of minimally invasive injectable therapeutic hydrogels to promote the neovascularization by delivering specific biomolecules constitute a promising approach to enhance blood flow. The aim of the research was to propose a novel approach in developing a multilayer hydrogel based on collagen/collagen–gelatin nanoparticles (GNPs)/collagen-low molecular weight hyaluronic acid (LMWHA) "Figure 1".

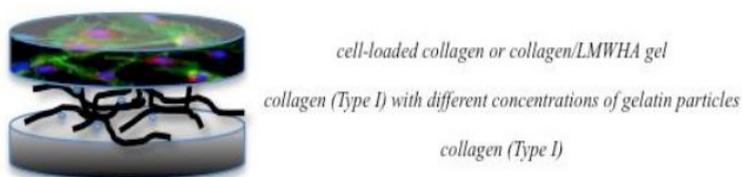


Figure 1: Schematic representation of the collagen-based system.

Three-layer systems were developed integrating a conventional method and an electrospray-based technique. A two-step desolvation method has been used for obtaining gelatin nanoparticles as a delivery vehicle of a model protein drug, bovine

serum albumin (BSA). The influence of BSA concentration on size, shape and release profile was also investigated. Small amplitude oscillatory shear tests and steady state shear measurements were performed on all the proposed systems in order to assess their viscoelastic properties. Finally, cell-particle interactions as well as different kinds of cell constructs were analyzed over culture time.

RESULTS AND DISCUSSION

The GNP preparation method allowed the production of uniform and spherical nanoparticles with a smooth surface. There were no differences on the morphology when BSA was added until a threshold BSA concentration. Nanoparticles with a mean size of 170 ± 36 nm were obtained. The BSA release profile showed a biphasic diffusion-controlled mechanism. All the proposed systems generally showed a gel-like and shear thinning behaviour, thus suggesting their injectability. A variation in cell morphology by varying BSA concentration over culture time has been observed. Furthermore, the number of viable cells adhering and proliferating increased over time for the different proposed system.

CONCLUSIONS AND/OR OUTLOOK

A multilayer composite hydrogel with tunable mechanical properties and able to support cell adhesion and proliferation was developed. Desirable gelatin nanoparticles were obtained by an optimized two-step desolvation method. BSA was successfully encapsulated and a threshold concentration for BSA encapsulation was found. Controlled release of protein was observed, showing a biphasic modulation characterized by an initial rapid release phase followed by a slower and prolonged release phase. The effect of BSA concentration on cell adhesion and proliferation was evaluated.

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COLLAGEN-HONEY FILMS: A NOVEL MATERIAL COMBINATION FOR THE MANAGEMENT OF WOUNDS

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Keywords: Collagen, honey, wound healing, wound dressing

INTRODUCTION

In 2015, the global wound care market has been estimated at over \$14 billion, with antimicrobial dressings and bioengineered skin and skin substitutes accounting for over 10% and 3% of this total respectively [1]. Indeed, wound management represents a challenging field, both in research and product development and in clinic, where patients present with an array of wound types. In particular, infection is a common problem in wound healing; this can result in reduced healing rates, prolonged hospitalization time and increased care costs.

The focus of this study is in the development of a novel dressing comprising collagen and honey which is able to actively promote healing while preventing infection in a variety of wound types. Collagen presents several properties that are desirable for a wound dressing: strong biocompatibility, weak antigenicity, biodegradability, in addition it can terminate the chronic state of a wound. [2, 3, 4]. The honey component exhibits anti-inflammatory and anti-bacterial properties, while also allowing for manipulation of wound pH [5, 6, 7]. Herein the combination of collagen and honey is investigated for the design of a novel bioactive wound dressing film able to actively promote healing. The films are intended to adhere and conform to the wound site and to degrade in contact with wound exudate.

RESULTS AND DISCUSSION

The fabrication process can be categorised in two main stages: the first the preparation of the suspension and the second the drying stage. The suspension is prepared by swelling of Bovine Type I Collagen (Sigma Aldrich) in acidic environment, followed by 1 hour of homogenisation, during which Manuka Honey New Zealand source) is added in different collagen/honey (C/H) weight ratios: C:H=5:1; C:H=5:2 and C:H=1:1. The resulting suspensions are poured into hydrophobic glass moulds (15cm x 7.8cm) and dried in a convection oven at 37°C to create thin homogeneous films with thicknesses of approximately 15µm. The materials are characterised for homogeneity, degradation and honey release kinetic, mechanical properties and the cell response of human skin fibroblast (BJ-ATCC CRL-2522) using AlamarBlue and colorimetric viability assays.

The homogeneity of the films is assessed through FTIR spectroscopy and scanning electron microscopy. Degradation studies performed at 37°C in saline buffers at pH levels ranging from 7.3 to 9.0 reveal that the films may have similar behaviours in different chronic wound environments. All films retain their integrity for up to 7 days but the presence of honey accelerates the degradation. This result is supported by honey release data showing that the majority of the honey present in the sample is released within 8 to 12 hours.

Tensile tests were performed on the samples in dry, extra-dry and wet conditions. In all cases the presence of honey did not have a significant effect on the mechanical properties. Water is a plasticizer and dry films are stiffer and stronger than wet ones. The ultimate tensile strength of dry and extra-dry films is of the order of 150 MPa up to 10 times higher than that of wet ones. However, wet samples elongate 3 times more than the dry ones and 10 times more than the extra-dry ones, before fracture. Indeed, fibrils alignment is observed in samples tested in wet conditions, suggesting that this contributes to the overall film deformation. All combinations present good handling properties which is positive for the final application. Initial cellular work suggests that the present combinations of collagen with honey provide good cell adhesion, viability and proliferation rate compared with the control culture well, over 7 days of culture.

CONCLUSIONS

We have developed homogeneous collagen-honey based films that exhibit relatively large tensile strengths (150 MPa) and degradation times in wet environments of the order of a week. During degradation these films release the honey and collagen

that can have a therapeutic effect. The films are transparent and support fibroblast proliferation. They may provide a new option to address a range of chronic wounds. In particular they may be applied in low exudating wounds and where conformability and wound site security is required.

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THE STUDY AND EVALUATION TO USE ECO-FRIENDLY SURFACTANT TO PREPARE DECELLULARIZED MATRIX FROM SMALL INTESTINAL SUBMUCOSA FOR NERVE REGENERATION

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Keywords: Decellularized, extracellular matrix, alkyl polyglucoside, surfactant, small intestinal submucosa.

INTRODUCTION

Decellularized extracellular matrix (DEM) is one of materials to remove cells from tissue or organ. The process leaves functional proteins and the complex structure of tissue scaffold. DEM have been widely used in tissue engineering/regenerative medicine applications [1]. Currently, decellularization methods often include detergents, such as Triton-X 100 and sodium dodecyl sulfate (SDS). SDS is one of the most commonly used detergent for decellularization, which is also a very powerful reagent for removing cells from native ECM. However, SDS have been reported could damage ECM structure through decellularization process. SDS not only exhibits cell toxic but also harmful for environment. In the study, we developed a novel decellularization process by using an eco-friendly surfactant to replace SDS. This process was not only efficient to remove cells but also reduced structural damage. It reserved

more functional proteins in DEM and enhanced the benefits for tissue engineering/regenerative medicine applications. Alkyl polyglucoside (APG) was used as a gentle detergent to treat small intestinal submucosa. APG is biodegradable, environmental friendly, and not harmful to human body [2]. It's also a green detergent in interfacial chemistry. This decellularization process was patented in Taiwan (Certificate No: I533860) [3]. Decellularization process by APG could reduce the problems of protein denaturation, residual antigen and structural damage of DEM. DEM could provide 3D culture space composed of scaffold and functional proteins to mimic for real tissue culture condition. In this study, we are going to conduct decellularization process on porcine small intestinal submucosa. Decellularized small intestinal submucosa (dSIS) was evaluated cell remained and biocompatibility after decellularization. We also performed recellularization by different cell line co-culture with dSIS for discovering the potentiality on tissue engineering/regenerative medicine applications.

RESULTS AND DISCUSSION

The results of DAPI staining and DNA content indicated that there was no cell remains on dSIS. It means that APG is very effective for removal of cellular components. Compared to other detergent, APG preserved more GAG and functional proteins content. Therefore, APG could be a candidate surfactant for SDS replacement. Cultivation of L929 cells with extraction medium. Results of cell viability and apoptosis detection indicated dSIS was no significant cytotoxicity. dSIS also exhibited good mechanical properties. Recellularization by different cell line on dSIS, we discovered cell line from central nervous system could attach and proliferation on dSIS surface. That indicated dSIS could be applied to neuron regeneration.

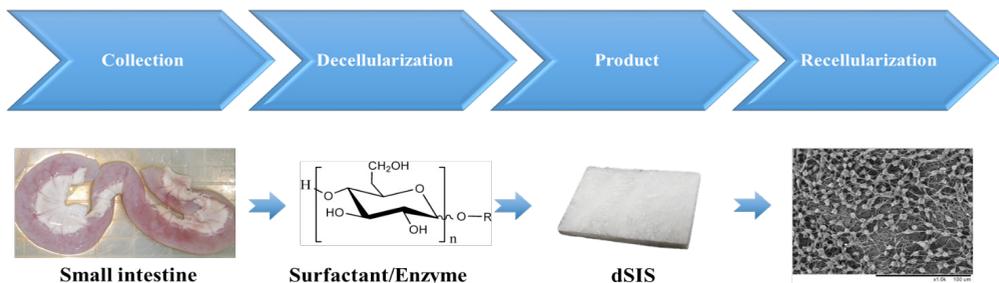


Figure 1: The flowchart of de-cellularized and re-cellularized process of APG-treated small intestinal submucosa.

CONCLUSIONS AND/OR OUTLOOK

Nowadays, being eco-friendly is a public issue in the world. In this study, we used a green surfactant in decellularization process. APG is not only harmless for human body, but also proved to reduce the damage on DEM. DSIS displays several benefits for tissue engineering/regenerative medicine applications, such as 3D infrastructure providing, functional protein content preserving, great mechanical properties and biocompatibility. We also discovered that DSIS could be a niche for neuron attaching. Summarizing the results, we suggested DSIS with the potentiality for neuron regeneration.

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SILICA NANOPARTICLES CONTAINING ZINC FOR CANCER THERAPY

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Keywords: mesoporous, silica, nanoparticles, cancer, zinc, biodegradable

INTRODUCTION

Zinc oxide nanoparticles (NPs) have emerged as a promising class of anticancer material, due to their selective toxicity against cancer cells.^[1,2] Whilst the detailed mechanism remains unclear, this anticancer selectivity is suggested to be related to the release of zinc ions as driven by the acidic microenvironment around cancer cells. However, the uncontrolled burst release of zinc makes ZnO NPs unsuitable for direct *in vivo* application, drug delivery systems with better controlled zinc release capabilities are needed. Here we report the design of a pH-stimuli, biodegradable silica based nano-platforms for delivery of zinc for cancer treatment. The NPs morphology, pH effect on zinc release rate, cytotoxicity on breast cancer cells and transmission electron microscopy (TEM) cell uptake study were carried out.

RESULTS AND DISCUSSION

In vitro ion release study

A faster zinc release was observed in acidic artificial lysosomal fluid (ALF, pH 4.5) than in MEM cell culture medium (pH 7.4), data not shown. In solution, protons can free Zn²⁺ from NPs by replacing Zn²⁺ from silica matrix or breaking down Zn-O bonds. Faster zinc release is therefore expected in lower pH environment. The zinc release rate can also be tuned by engineering the morphology of NPs. For

instance, mesoporous silica NPs (MSNPs) of a 15 times larger surface area than dense silica NPs (d-SNPs), showed a much faster zinc ion release rate.

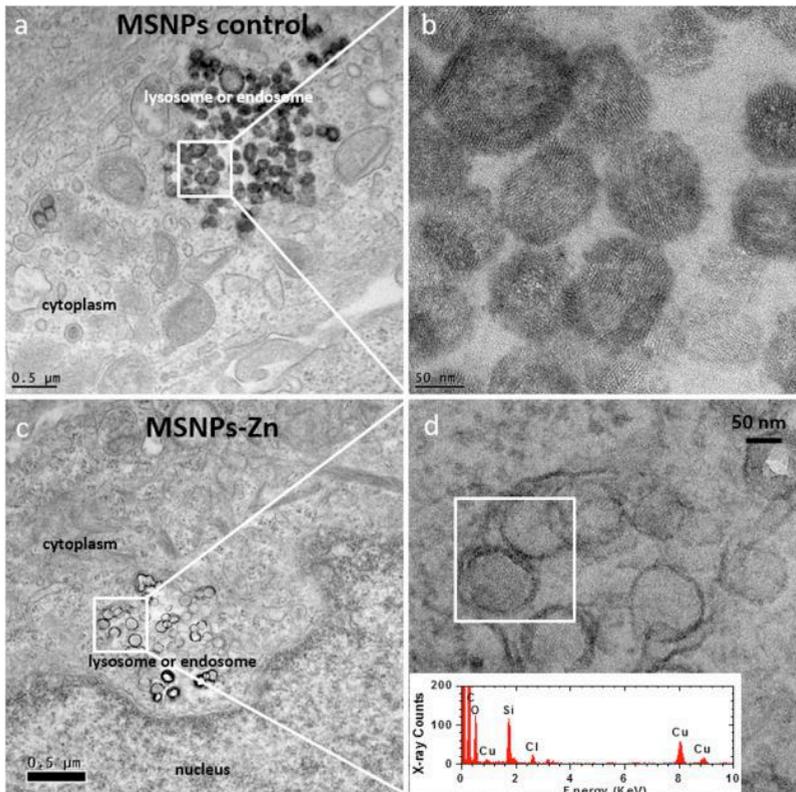


Figure 1: TEM cell uptake analysis of MSNPs (with/without Zn) after cell exposure for 24 h. Bright field TEM (BFTEM) image (a) shows the presence of MSNPs inside a lysosome/endosome like vesicle, which do not show significant morphology change as revealed by higher magnification TEM imaging (b). In contrast, BFTEM image (c) shows MSNPs-Zn has transformed to hollowed out structures inside a lysosome/endosome like vesicle. A higher magnification TEM image (f) further shows the morphology of degraded NPs. Chemical composition of the transformed NPs was analysed by scanning transmission electron microscopy energy-dispersive X-ray spectroscopy (STEM-EDX), collected from the boxed area in (d). The insert shows the corresponding EDX spectrum.

Cytotoxicity

Whilst d-SNPs had very low toxicity against breast cancer cells due to the slow zinc release, MSNPs containing Zn (MSNPs-Zn, data not shown) showed promising selective anticancer properties. MSNPs Zn caused significant toxicity against MCF-7

and MDA-MB231 breast cancer cells, without affecting MCF-10a healthy cells, at NPs concentrations from 75-125 $\mu\text{g}/\text{mL}$. This selective toxicity against cancer cells could be attributed to a faster metabolism in cancer cells resulting in increased uptake of particles and increased metabolism of Zn^{2+} ions, or a higher zinc release in the more acidic microenvironment of cancer cells. TEM cell uptake After 24 h, MSNPs-Zn have transformed to hollowed out structures (Fig 1 c & d). STEM-EDX analysis indicates no zinc content in remaining structures, suggesting successful intracellular zinc release. In contrast, no significant morphology change of MSNPs without Zn inside cells was observed. This result demonstrates the incorporation of Zn can change original biopersistent silica NPs to biodegradable nanomaterial.

CONCLUSIONS AND/OR OUTLOOK

Our results suggest silica NPs based drug delivery systems containing zinc are promising candidates for zinc anticancer treatment. NPs have shown a pH dependant zinc release behaviour, i.e. faster zinc release in lower pH environment, which is a huge advantage for selective killing of cancer cells, considering the extracellular environment of cancer cells is more acidic than normal cells. The toxicity behaviour which is related to zinc release rate, can be further tuned by engineering the morphology of NPs. Whilst dense NPs showed very low toxicity against breast cancer cells for their slow zinc release, mesoporous NPs induced significant toxicity against MCF-7 and MDA-MB231 cancer cells, and leaving healthy cells MCF-10a unaffected within the dosage range of 75-125 $\mu\text{g}/\text{mL}$. This is attributed to a much larger surface area of mesoporous NPs than solid NPs, hence a faster release of zinc ions and also breaking down of silica network, as indicated both by *in vitro* ion release and TEM cell uptake studies.

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NONTOXIC PREVENTION AND TREATMENT OF CANCER

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If a malignant tumour is diagnosed, treatment will follow. If the tumour is found in an early stage, the tumour can be removed by a surgical procedure. When surgical intervention is no longer an option, chemotherapy and radiation will be the choice today. Usually fluorouracil (5-FU), cisplatinum, taxol, vincristine and their derivatives are in use. Genetic approaches in association of the mutation status of BRAF oncogene are new favourites. They will delay some trouble but will not heal the patient. Unfortunately, one of the side effects of today's treatment is the destruction of the body's own defence system. Nontoxic treatment, however, offers better results.

A new option in cancer treatment is the use of artemisinin derivatives. Artesiminin is an extract of the plant *artemisia annua*, currently in use for the treatment of malaria. Dihydroartemisinin (DHA) is the most active preparation of artemisinin. A Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer has been demonstrated..

Recent research shows that an effective stimulation of the immune response can be achieved by the iron binding protein Lactoferrin . It has been demonstrated that cancer cells have a preference for iron molecules, which will be absorbed in high level. DHA reacts with high concentrations of iron. Then cancer cells will disappear by apoptosis, necrosis and fibrosis .

The combination of dihydroartemisinin and iron loaded lactoferrin seems to be 100 x more effective than each of the components alone. Alpha lipoic acid supports anticancer activity.

During a recent medical trial it has been demonstrated that after 3 weeks of treatment 40% of a breast cancer will show necrosis and fibrous replacement.

3 month of treatment will probable result in a total disappearance of all kind of cancer. Results and a new protocol for the treatment of cancer will be presented.

APPLIANCES OF INNOVATIVE TECHNOLOGIES WITH ADULT STEM CELLS AND NANOMATERIALS

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Congenital, genetic, traumatic and degenerative disorders, and the increased average of life expectancy has its problems in human people and, also, among pets and others animals (horses, animals for slaughter).

Age-related problems, the great linked life between human and pets (home life), and the mobility (globalisation) due to international transports draw a new concept of pets. Pets are really family members, they can travel with family and have a specific role affective, stabilisation and armonisation of family life, specially for kids.

This, in last years, has produced an increased number of diseases that can be exchanged between human and animals (zoonoses) that have to be verified.

There is contamination of human illness to pets, always related to alimentation. Pets eat often the same food of owners. For this reason we can find in pets, an increased value of medical and dental problems (caries, parodontal problems, articular diseases).

This for owners and veterinarian is too complicated to manage and the recover, with a considerable gain of economic and sentimental value is a desired outcome. The linked life to humans, in towns, has produced also, an increased number of traumatic problems related to accidents (cars) or fighting with others animals.

In our work we can bring forward bones, parodontal, dental and oral recover in

veterinary dentistry and veterinary medicine-geriatrics to improve the quantity and quality of craniofacial tissues.

The stem cell applied in regenerative medicine, can modulate the repair of damaged tissues, used alone or in combination with nanobiomaterials, as a scaffold in order to create a suitable 3-D environment helpful in restoring the anatomy and the functionality of organs and oral tissues. This is an innovative appliances versus traditional therapies for the treatment of numerous injuries of, and it becomes the “gold standard” therapy for many illnesses in animals and we propose launching this pilot scheme, it can be a protocol of treatment also in human (within the limits of the law).

Fusing the most remarkable technologies, nowadays producers are manufacturing industrial materials and devices that can help to restore always more the functionalities of damaged body, towards self-regeneration. This innovation is an exciting scientific challenge with the goal of producing bioinspired materials integrated with living tissues. A clinical evaluation was carried out with imaging, histological and biochemical analyses in a follow-up. The data obtained will be correlated with the standard intervention procedures and analyzed in order to draw up a farsighted perception of new lines of research.

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NOVEL NANOSTRUCTURED THIN FILMS FOR BIOMEDICAL APPLICATIONS

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Keywords: Bone apatite-like coatings; zirconium oxide; magnetic nanoparticles; antibacterial coatings

INTRODUCTION

When osteoarthritis occurs, total joint arthroplasty (TJA) is the most frequent medical treatment. In TJA, the natural joint is removed and replaced by a metallic implant in which the joint is represented by a soft plastic insert (usually UHMWPE) slides against a metal or ceramic counterpart. On the new-generation uncemented implants, a hydroxyapatite (HA) coating is generally deposited on the metal surface in contact with bone, in order to improve the short-term and long-term fixation of the device in the surrounding bone. TJA is generally successful; however, about 15–20% of the installations could fail after about 15 years. Then a revision surgery with high cost and conceivable troubles for the patient is necessary. The main reason for implant failure is aseptic loosening of the implant, i.e. the mobilization of the implant without infection. In turn, the aseptic

loosening is strongly correlated with the formation of wear debris from the UHMWPE component and with delamination of the mechanically weak HA coating. In addition, peri-prosthetic joint infection (PJI), is one of the major complications and aetiologies of implant failure after TJA, associated with substantial financial burden on the healthcare system and significant physical and psychological morbidity on patients [1]. The biological and mechanical properties of conventional implant materials can

be significantly improved by tuning their surface characteristics by the addition of a suitable coating layer. Several coating techniques have been proposed to improve the performance of implantable materials; among them, plasma spraying (PS) plays a primary role; however several concerns mainly related to poor mechanical properties and inhomogeneity of thick (at least 30 μm) plasma spray coatings, led researchers to consider alternative deposition techniques, able to produce thin films (less than 5 μm), more adherent, homogenous and dense than PS coatings. Our research is focused on the possibility to realize nanostructured, well-adherent, dense and homogenous thin films, even at room temperature (i.e. enabling the coating of heat-sensitive materials such as plastics), through an innovative pulsed electron deposition technique. More specifically, we are developing: a) biomimetic calcium phosphate films able to boost bone regeneration and implant fixation, b) magnetic calcium phosphate films able to reduce bacterial adhesion and biofilm formation and c) hard ceramic coatings with the aim of reducing the wear of the soft plastic component in a TJA.

RESULTS AND DISCUSSION

a) Bone apatite-like thin films were obtained by a biological apatite target. FTIR, XRD and XPS analyses confirmed the preservation of the structure and composition of the starting material into the film. Results were compared to those of stoichiometric HA. Morphological and micro-structural modifications upon annealing at different temperatures up to 600 $^{\circ}\text{C}$ were investigated. The adhesion and mechanical properties of these films were also assessed.

b) With the aim of fabricating novel magnetite/calcium phosphate-based films exhibiting antibacterial properties, the pulsed electron deposition technique was shown to yield nanostructured magnetic coatings with surface composition and magnetic moment similar to the deposition target [2]. More specifically, the quantitative XPS analysis strongly suggested the presence of a dominant magnetite phase at the surface, together with other satellite iron oxide phases. According to this scenario, the EFM (Electrostatic Force Microscopy) showed the presence of randomly-distributed surface areas exhibiting inhomogeneous charge distribution, which in turn indicates that a compositional variation occurs over distances of a few micrometres or less. The STM analysis supported these findings revealing $< 1 \mu\text{m}^2$ large islands featuring homogeneous small-bandgap states that suggest the presence of Fe_3O_4 -rich regions. Finally, preliminary bacterial adhesion tests suggested that magnetite/HA films were able to hamper the bacterial adhesion capability in comparison with the stoichiometric

HA coatings.

c) Yttria-stabilized zirconia (YSZ) thin films were directly deposited onto the surface of both the plastic and metallic component of a TJA and the wear of UHMWPE investigated. YSZ films exhibited a fully cubic structure, with densely packed grains, whereas mechanical tests showed that hard, tough and well-adherent films were deposited, despite the high degree of mechanical mismatch between the film and the substrate (Figure 1) [3]. Finally, suitable mesenchymal stem and osteoblast cells adhesion, proliferation and viability were observed, suggesting good biocompatibility of the nanostructured zirconia films [4].

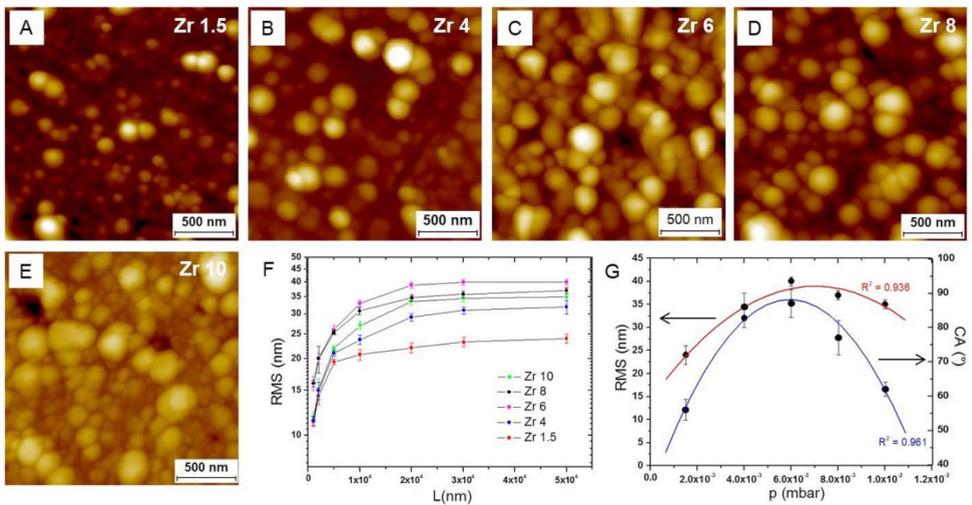


Figure 1: A-E) Representative AFM topographical images ($2 \times 2 \mu\text{m}^2$) of zirconia films deposited at oxygen pressure values ranging from 1.5×10^{-3} to 1×10^{-2} mbar; F) plot of the log(RMS) vs lateral image size; G) trend of RMS roughness and contact angle in serum vs. working gas pressure.

CONCLUSIONS AND/OR OUTLOOK

Nanostructured thin films can provide novel and higher properties to common materials used in implantable devices, leading to new-generation total joint implants less subjected to wear and aseptic loosening, bacterial adhesion and proliferation as well as more prone to integrate with the surrounding bone can be realized. This improvement will lead to a decrease of the number of implant failure and saving cost

for the Healthcare System as well as troubles for the patients.

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SURFACE MODIFICATION OF ADDITIVE MANUFACTURED TITANIUM WITH CAP, AG NANOPARTICLES AND ULTRATHIN HA COATING

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Keywords: titanium, hydroxyapatite, calcium phosphate, silver, additive manufacturing, RF-magnetron sputtering, electrophoretic deposition

INTRODUCTION

The use of porous lattice structured titanium-based implant materials for bone contact has been gaining ground in recent years. Electron beam melting (EBM) is a rapid prototyping method allowing for manufacturing of the porous implants with highly defined external dimensions and internal architecture [1]. Titanium alloys additively manufactured implants can also successfully replicate the complex microstructure of the substituted bones by integrating porous sections into monolithic implants improving the implant integration process and its long term stability in the body. Titanium-based materials are at best bioinert, so after implantation they are encapsulated by fibrous tissue. And different surface modification methods are commonly applied to improve the surface biocompatibility. Compared with bulk calcium phosphate (CaP) coatings, nanoscale CaP particle ones (CaPNPs) are found to show better biocompatibility [2]. It is also found that silver nanoparticles (AgNPs) show efficient antimicrobial properties due to their large specific surface area which enhances the release of silver ions [3]. In this research, CaP and Ag particles are used in the implant surface coating. Electrophoretic deposition can be used to fabricate well-distributed particles layers

on surfaces [4]. Radio-frequency (RF-) magnetron sputtering is a physical deposition method that allows the fabrication of thin, dense and well-adherent hydroxyapatite (HA) coatings with adjustable composition, crystallinity and improved biocompatibility [5]. The aim of this study is to generate the AgNPs and CaPNPs assembly and HA coatings on the as-manufactured Ti surfaces via electrophoretic deposition and RF-magnetron sputtering, and investigate the properties of formed composites.

RESULTS AND DISCUSSION

All used samples were fabricated using ELI titanium powder in ARCAM A2 EBM® machine at Mid Sweden University, Sweden. Figure 1 illustrates examples of titanium lattice and planar samples fabricated by additive manufacturing using electron beam melting.

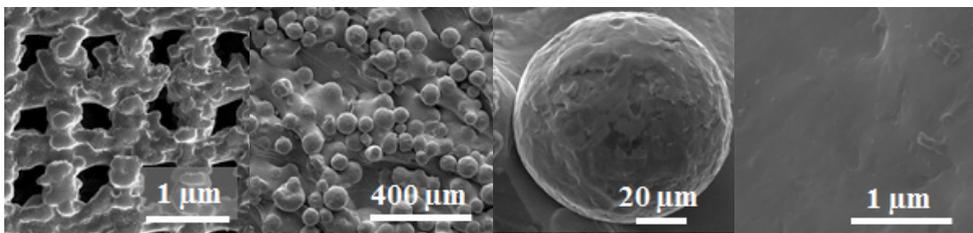


Figure 1: Scanning electron microscopy images of the as-manufactured titanium-based sample surface morphology

The titanium substrates were subjected to four different modification procedures, as outlined below: (i) the preparation of a nanocrystalline 1000 nm-thick HA coating by RF-magnetron sputtering (Figure 2 a); (ii) the electrophoretic deposition of AgNPs onto the as-manufactured (?) titanium substrate (Figure 2 b); (iii) the electrophoretic deposition of CaPNPs onto the as-manufactured (?) titanium substrate (Figure 2 c); (iv) the electrophoretic deposition of CaP/AgNPs onto the as-manufactured (?) titanium substrate (Figure 2 d).

CaPNPs were synthesized by precipitation in the presence of poly(ethyleneimine) (PEI) [6]. Wet chemical synthesis and characterization of poly(vinylpyrrolidone) (PVP)-stabilized AgNPs were reported elsewhere [7]. The modified titanium substrates were studied in respect to its chemical composition and surface morphology, water contact angle, hysteresis, and surface free energy.

The deposited HA coating was homogenous and revealed a regular grain-like

morphology, which is typical for RF-magnetron sputter deposited coating (Figure 2 a). The structure and morphology of the HA films deposited by RF-magnetron sputtering can be successfully controlled by deposition parameters. The deposition parameters (power density, voltage, gas pressure, and target – substrate distance, deposition time and rate, bias and temperature applied to the substrate) can be adjusted to fabricate unique phase of crystalline and stoichiometric HA coatings [8]. The results of microphotography analysis showed that the AgNPs, CaPNPs and combined CaP/AgNPs were homogeneously distributed over the surface (Figure 2 b-d). Following parameters affecting the deposition were varied in present experiments: deposition time, potential, and temperature [6].

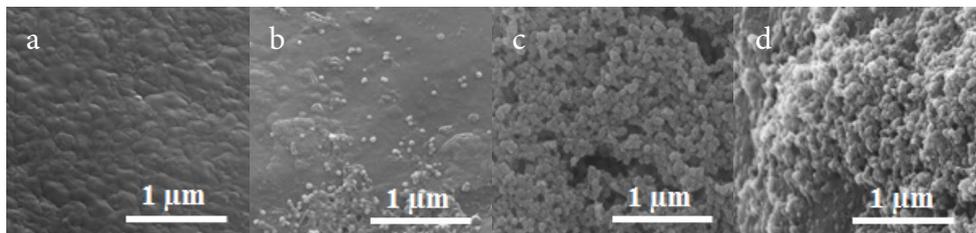


Figure 2: SEM image of the electron beam melted titanium coated with thin HA film (a), AgNPs (b), CaPNPs (c) and CaP/AgNPs

RF-magnetron sputtering allowed to produce the HA coating with a lower water contact angle and a higher free surface energy compared to uncoated Ti substrates. Thus, it is concluded that the increase in the surface energy, in particular, the increase in the polar component of the surface energy as well as the change of the surface chemistry, surface hydrophilicity and water contact angle can improve interaction between surfaces and cells. The titanium-based scaffolds covered AgNPs, CaPNPs and CaP/AgNPs assemblies exhibited a super-hydrophilic surface because the water contact angle was $< 5^\circ$. Therefore, the modified titanium-based substrates are a prospective material for biomedical applications.

CONCLUSIONS

The titanium-based samples were fabricated with controlled porous or dense structure by electron beam melting process. Sample surface modification was carried out using different methods. RF-magnetron sputter deposition and electrophoretic deposition were applied to modify the surface biocompatibility. Thin HA nanostructured films

were fabricated by RF-magnetron sputtering. As-manufactured by EBM titanium surfaces were electrophoretically coated with CaPNPs and AgNPs. Coated Ti surfaces show reduced water contact angle, increased surface free energy and its polar component as compared to as-manufactured ones.

ACKNOWLEDGEMENTS

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THIN NANOSTRUCTURED BIOACTIVE FILMS OBTAINED BY MEANS OF LASER DEPOSITION TECHNIQUE.

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Keywords: Pulsed Laser Deposition, Thin films, Bioactive materials, Bioglass.

INTRODUCTION

Production of biomedical devices requires the fulfilment of several parameters, such as the mechanical strength of bulk materials, their non-toxicity and bioactivity. In particular, biological response of the device is mainly related to the interaction between the its surface and biological surrounding [1]. In this contest, the coating of titanium implants by thin bioactive films allows to obtain materials suitable for in-bone implants due to the high mechanical strength of the metal and the osteo-conductive properties of the coating. Among the deposition techniques used to obtain thin bioactive films, Pulsed Laser Deposition (PLD) presents several advantages such as congruent transfer of the target composition to the coating, the possibility to control film's thickness, degree of crystallinity, adherence and surface roughness, simply varying experimental parameters such as the deposition temperature and time, target-substrate distance, laser wavelength, pulse duration or repetition frequency. Moreover, by PLD it is possible to deposit thin films on substrate of different composition, shape and dimension. The efficiency of the nanosecond ablation and deposition technique has been proved by growing thin films of bioglasses and bioceramic of different composition. Finally the possibility of obtaining bioglasses composite with improved mechanical properties has been checked combining bioglass with fullerite and reduced graphene oxide.

RESULTS AND DISCUSSION

The laser ablation and deposition experiments have been carried out by means of a nanosecond laser source Nd:YAG ($\lambda=532$, $t=7$ ns, repetition rate=10Hz). When a high energy laser source hits a solid, the local evaporation of the target in the laser spot takes place, with the generation of a plasma containing ions, atoms, molecules and target droplets. The laser induced plasma can expand and deposit on a substrate, allowing the formation of a thin film that can retain the target composition. Three bioglasses, synthesized by sol-gel methodology, with different composition have been used to deposit thin films on titanium substrate [2, 3]. Substrate-target distance, laser energy and substrate temperature have been varied in order to choose the optimal deposition conditions. It has been showed that with increasing the substrate temperature, compact nanostructured films, retaining target composition and crystallinity have been obtained. Biocompatibility and cell-friendly properties of the deposited film have been tested by using human colon carcinoma CaCo-2 cells. Finally, the possibility of tailoring the mechanical properties of the deposited films has been exploited using composite targets (RKKP+RGO and RKKP+C60). Films obtained by doping RKKP with fullerite show Raman features typical of graphene-like domains. On the other hand, it has been observed that during nanosecond ablation of RKKP+RGO target, graphene oxide can be transferred on the substrate and can form a network inside the thin deposited film (Figure 1).

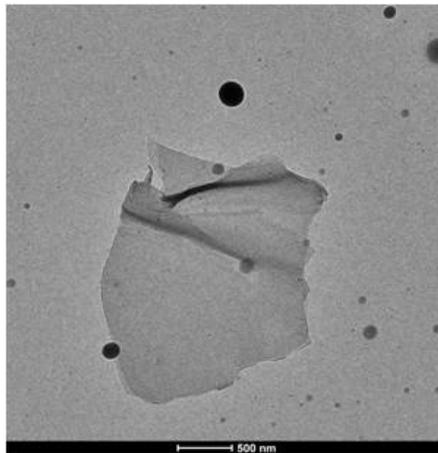


Figure 1: TEM image of the early step of formation of the RKKP+RGO film deposited by nanosecond laser source.

We suggest that the improved hardness measured for RKKP+C60 and RKKP+RGO films can be related to the presence of graphenic network inside the bioglass .

CONCLUSIONS AND/OR OUTLOOK

PLD technique has been used to deposit thin nanostructured films of bioglass of different composition. Films retaining the targets stoichiometry, with the relative proportion of all elements preserved, have been obtained. It has been showed that films are cell-friendly substrates for the cells adhesion, growth and differentiation, and can represent a new potential cell delivery system that may be used in tissue engineering applications and in the future regenerative medicine protocols. Finally, the possibility to improve film hardness by doping RKKP bioglass with RGO or fullerite, has been exploited.

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GREEN SYNTHESIS OF IRON OXIDE NANOPARTICLES AND THEIR POTENTIAL APPLICATION IN COMPOSITE FILM COATING OF BONE-RELATED IMPLANTS

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Keywords: iron oxide, hydroxyapatite, laser ablation in liquid

INTRODUCTION

The synthesis of iron oxide nanoparticles (Fe_xO_y NPs) has become an important area of research because of the growing scientific and technological interest for their potential applications. Between iron oxide nanomaterials, magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$) are especially promising for applications in nanomedicine and tissue engineering because of their biocompatibility and low toxicity when in contact with the human tissues [1]. Nowadays, a large number of physical and chemical methods for the production of NPs exist; among them, the Laser Ablation in Liquid (LAL) is one of the simplest, cheapest and cleanest method for the synthesis of Fe_xO_y NPs, because it does not require chemical precursors and nanoparticles are directly obtained in water as a stable colloidal system [2].

In this work, Fe_xO_y NPs have been produced by LAL technique in water, using a Ti:sapphire laser source ($\lambda = 800$ nm, $\tau = 100$ fs, repetition rate = 1 kHz). Afterwards the Fe_xO_y NPs have been mixed with hydroxyapatite (HA) powder and pressed in order to obtain a black pellets. The latter has been the target material for a second laser process, the Pulsed Laser Deposition (PLD). For the deposition a doubled Nd:YAG laser has been used ($\lambda = 532$ nm, $\tau = 10$ ns, repetition rate = 10 Hz). The

results of these processes have been composite HA& Fe_xO_y thin films, with potential application as film coating of bone-related implants. In Figure 1, a scheme of the two laser processes is shown. The characteristics of the obtained films have been investigated with spectroscopical and microscopical analysis.

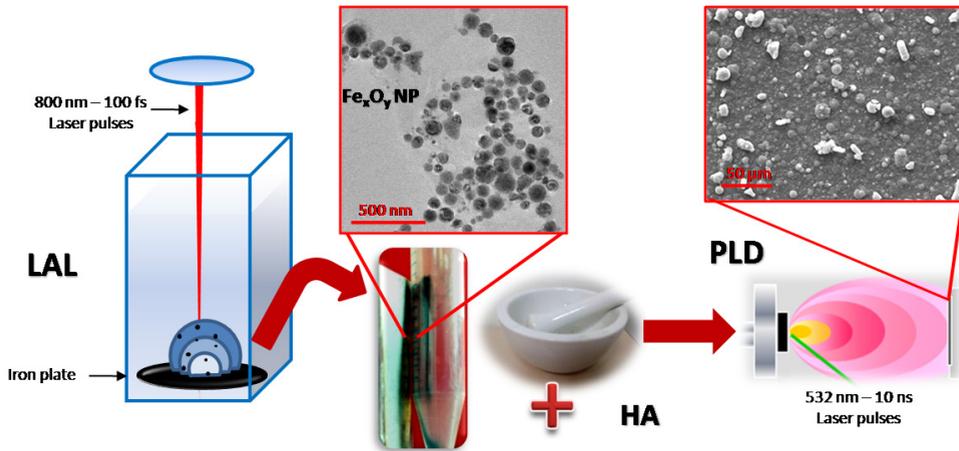


Figure 1: Scheme of LAL for the production of Fe_xO_y NPs and PLD for the deposition of HA& Fe_xO_y thin films

RESULTS AND DISCUSSION

Different phases of iron oxide nanoparticles have been obtained by the Laser Ablation in water of the iron target. The maghemite and magnetite phases have been detected by Raman spectroscopy, X-Ray diffraction (XRD) and high resolution transmission electron microscopy (HR-TEM) analysis.

The PLD process of HA&Fe_xO_y target, conducted at different deposition temperatures, leads to the production of composite films. HR-TEM gives information on the first steps of film growth; it has been possible to reveal the presence of several crystalline domains with different interplanar distance, attributable to HA, maghemite, magnetite and hematite phases. Scanning electron microscopy (SEM) reveals that all the films are characterized by a compact background of nanoparticles, moreover on the film obtained at higher deposition temperatures it is possible to note the presence of bigger particles, due to the aggregation and crystallization of the smaller ones. Indeed,

by increasing the deposition temperature it is possible to improve the film crystallinity, as XRD reveals. Furthermore film crystallinity affects its mechanical properties [3].

CONCLUSIONS AND OUTLOOK

Two different laser process have been applied in this work. LAL has allowed to produce “green” Fe_xO_y NPs. Afterwards, Fe_xO_y NPs in a mixture with HA have been used as starting materials for the deposition of bioactive film coating for bone-related implants. Considering that nanostructured film coating can improve the bioactivity of the implant and that the presence of Fe_xO_y NP benefits cells growth, we expect that successful implant could be produced with our materials and techniques.

Magnetism measures and bioactivity tests will be conducted on these films to prove their effectiveness.

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DESIGNING MAGNETIC NANOMATERIALS FOR BIOMEDICAL APPLICATIONS

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Design of magnetic nanomaterials for specific biomedical applications (e.g. Drug Delivery, Magnetic hyperthermia, MRI) means to control the matter at the nanoscale, correlating magnetic properties, micro and mesostructure and molecular coating. Taking into account all of these points, this chapter will focus on the design of magnetic nano-architectures for biomedical applications, including discussion on the main strategies for the synthesis and functionalization of magnetic nanomaterials. Various modern advances in construction of complex nanoarchitectures which are showcased for biomedical application are reviewed. These nanocarriers are typically of core-shell morphology and their shell may be composed of polymers, surfactants or mesoporous silica, which typically serve for embedding the therapeutic agents within their framework. Selectivity of the treatment is ensured through employing magnetic field-responsive homing of the nanocarriers to the therapeutic area, along with possibilities for alternating magnetic field hyperthermia-resulted treatment of the ill tissues. The induced hyperthermia may be therapeutically active through causing denaturation of biomolecules in the treatment area, or/and through mediating release of the cargo therapeutic agents. Targeted therapy is also augmented through specific functionalization of the nanoarchitectures with ligands-targeting the overexpressed receptors in the diseased tissue, along with devising nanocarriers whose drug delivery attribute functions upon exposure to externally applied stimuli (e.g. light irradiation) or to internally available biomolecules (e.g. enzymes).

BIOFUNCTIONALIZED ULTRA-SMALL SUPERPARAMAGNETIC NANOPARTICLES FOR BIOMEDICAL APPLICATIONS

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Keywords: Superparamagnetic nanoparticles; Biofunctionalization; Nanomedicine

INTRODUCTION

Over the last decade, nanotechnology has become more relevant in medicine. Among magnetic nanomaterials the future of iron oxide nanoparticles (IONPs) for clinical applications relies on their biocompatibility in moderate doses as well as their ability to be produced in a wide range of sizes and shapes with biofunctionalization potential. Additionally, they show great promise to serve as a cell tracking system in cell-based therapies, and to generate local temperature increases in the magnetic thermotherapy of solid tumours. Thus, the study and development of novel magnetic nanoparticles for biomedical applications is one of the key topics in the field of nanotechnology.

RESULTS AND DISCUSSION

and conjugated with FITC, as molecular model specimen. Nanoparticles were characterized by dynamic light scattering (DSL), transmission electron microscopy

(TEM) and X-ray diffraction analysis (XRD) and surface functional groups and composition were analysed by infrared spectroscopy (FTIR).

We assessed the biocompatibility of the magnetic nanoparticles carriers with biofunctional coating (FITC-conjugated) using a colon carcinoma cell line (CaCo-2) as human cellular model. Phase contrast, fluorescence and confocal microscopy analyses were performed to study nanoparticles up-take and internalization (Figure 1).

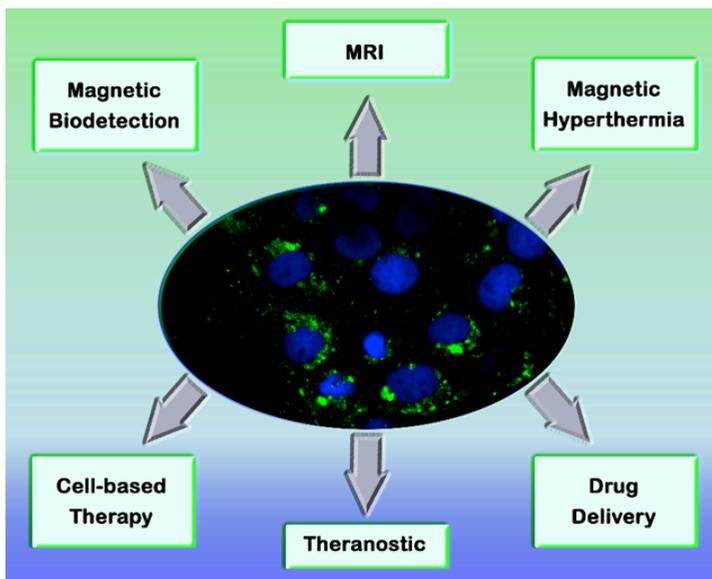


Figure 1: Magnetic nanoparticles internalization and biomedical applications

By transmission electron microscopy technique was investigated the effect of their internalization on ultrastructural features and intracellular compartments. Cellular growth and viability resulted unaffected following nanoparticles up-take and lack of toxicity was confirmed at transcriptional and translational level. Finally, even when used at high concentration, the cytotoxicity effect of the nanoparticles was not significant compared with control experiments.

OUTLOOK

All these results render the described so-synthesized superparamagnetic nanoparticles interesting potential candidates in nanomedicine applications for diagnostic and therapeutic tools.

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SCANNING SAXS MICROSCOPY STUDIES OF THE INTERFIBRILLAR PACKING IN BOVINE CORNEA

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Keywords: nanostructured biological systems, nanomedicine, X-scattering, imaging,

INTRODUCTION

X-ray scanning microscopy with both small (SAXS) and wide (WAXS) angle contrast, provides selective information at the nanometric and atomic scale, respectively, allowing two dimensional (2D) imaging with a lateral spatial resolution down to the micrometer scale.

In particular, Small Angle X-ray Scattering technique has found interesting applications in nanomedicine for the study of healthy or pathologic tissues from breast, brain, cornea, bone, etc., to detect structural changes at supramolecular level, and in some cases also to derive clear correlations between tissue changes and disease development or onset. In addition, scanning SAXS microscopy has become a suitable technique to quantitatively map the nanostructural heterogeneity of biological materials over extended areas [1], thanks to the recent progress in the development of X-ray microbeam sources combined with scanning methods for imaging [2].

A key issue in most of these experiments was the need for a high brilliance synchrotron radiation X-ray source to obtain high-quality scattering patterns, although a limited number of papers made use of laboratory systems mainly for bone and breast tissues analysis [3]. The possibility to use a laboratory X-ray source for SAXS studies in

nanomedicine is extremely important, because - in order to transfer this knowledge into a clinic - a room-sized system is mandatory.

We will show the application of a table top super-bright microfocus laboratory X-ray source coupled to a three-pinhole camera [4-5], which is used to investigate the supramolecular structure of soft matter in SAXS ex-situ experiments. In particular, the combination of the microsource brilliance and an original theoretical approach allows to restore diffraction features from SAXS profiles collected from low scattering biomaterials or soft tissues, reaching data quality compared to synchrotron radiation data. As relevant example we will show SAXS data obtained with the optimized set-up on bovine cornea treated with UV-crosslinking treatments studied by SAXS scanning microscopy.

RESULTS AND DISCUSSION

Here we investigate a bovine cornea treated with UV-CXL procedure by SAXS scanning microscopy by set-up a procedure able to derive the collagen supramolecular structure. The proposed methodology, based on the combination of statistical (Adaptive Binning and Canonical Correlation Analyses) and crystallographic (Pair Distribution Function calculation) approaches, demonstrates the possibility to observe structural variations across the tissues in room-sized laboratory, due to the chemical/physical treatments. The correctness of the adopted methodology is deduced by comparing synchrotron radiation data with table top X-ray microsource ones, besides the abundant data from literature.

Both synchrotron and laboratory data revealed:

- a decrease in the interfibrillar distance and in the shell thickness around the fibrils from the periphery to the center of the cornea; the central area coincides with the region (~10 mm) where the epithelium has been removed for the CXL treatment;
- no significant change in the diameter of the fibrils was measured across the explored area;
- the array of fibrils resulted packed according to a centered hexagonal symmetry.

The three major findings of our analysis, extracted on both laboratory and synchrotron radiation datasets, are validated by literature.

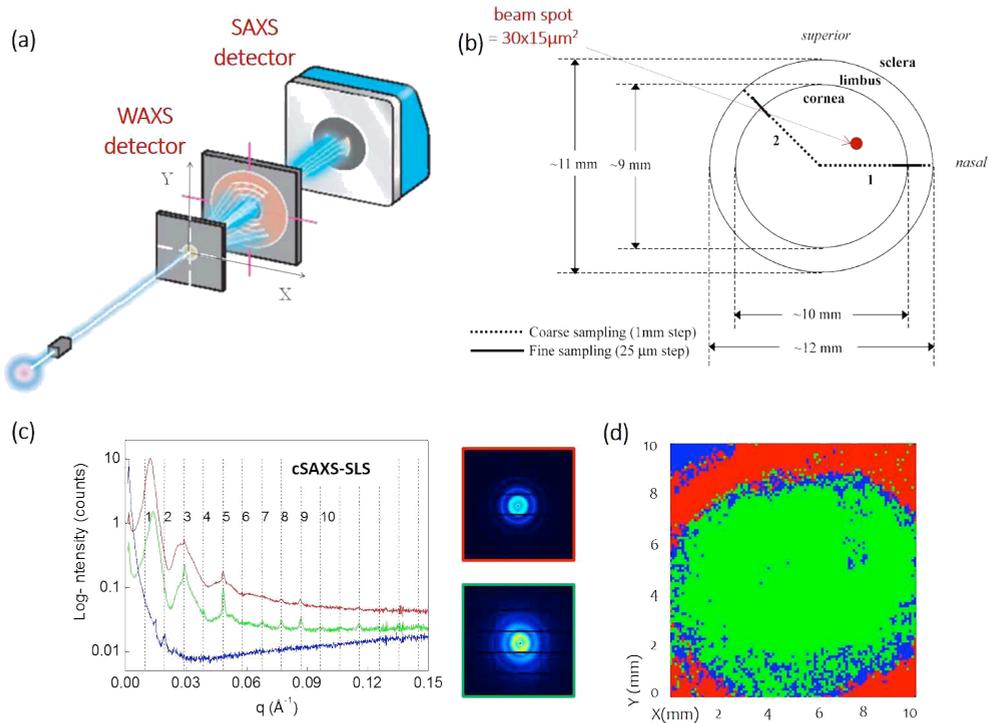


Figure 1: (a) sketch of the experimental set-up; (b) diagram of the cornea scanned area; (c) synchrotron ref data, as selected by Adaptive Binning from the synchrotron dataset: blue background, red and green SAXS profiles corresponding to the 2D SAXS data of the insets; (d) results of the canonical correlation analysis (CCA) on the explored area (synchrotron dataset), with red color corresponding to the red profile in (c) and marking the outer corona without epithelium layer and green color corresponding to the green profile in (c) in the center of the cornea.

CONCLUSIONS AND/OR OUTLOOK

The main aim of this work is to propose a procedure to investigate the collagen ultrastructure in cornea. Cornea tissue is a particularly suitable model of study thanks to the huge amount of results available in literature. The same cornea is explored by scanning SAXS microscopy with both a table-top and synchrotron X-ray microsource to obtain a direct comparison between datasets of different quality. Indeed, several studies already demonstrated the suitability of synchrotron radiation X-ray diffraction methods to obtain ultrastructure details and quantitative information on the molecular structure of cornea. Therefore, synchrotron data and literature are used

to validate the method here proposed and applied to inspect the lateral packing of the collagen microfibrils in the cornea ultrastructure and to prove that reliable data can be extracted also with laboratory instrumentation. The proposed methods can be applied to several type of natural and artificial biomaterials even in healthy and pathologic conditions and/or after chemical or physical treatments.

ACKNOWLEDGEMENTS

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THE SHAPE PROJECT - A NEW THEORETICAL FRAMEWORK OF THE MICROGRAVITY-CELL INTERACTION

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Defining the theoretical model that can explain the interaction between the gravitational field and living organisms is a needed and urgent task of our time. The definition and validation of such a theoretical framework is not only a prerequisite for the development of the Space Biomedicine and Exobiology, but is likely to have important consequences on the Biology and Medicine study. Indeed, it can be so, to the extent that it allows reconsidering the foundations of the latter in the role played by physical constraints in the control of processes and vital functions of complex living systems.

Investigating the extent to which the effect induced by microgravity on living structures is dose-dependent, and within what time limits it can be defined reversible, is one of the most important related issues. Refining the experimental model and scientific methodology inherent in the study of microgravity is mandatory, in order to realize the abovementioned aims. In particular, it is necessary to validate a new experimental protocol for the study of gravitational effects in three dimensions. This aspect is of fundamental importance since the control and the determination phenotypic evolution cell and tissue depend not only from the cell itself, but also and mainly by interactions with the stroma and the structures of the microenvironment. This cross talk between the cell and the microenvironment in three dimensions is not so far never been investigated in the specific conditions represented by microgravity.

We use the Atomic Force Microscope for the morphological and mechanical

characterization of cells exposed to a microgravity environment. Preliminary data suggest that the microgravity weakens (in terms of yielding a reduced stiffness) the interactions between cells and substrate. Such interactions are largely modulated by the cell class of integrins, i.e. proteins that modulate the shape and anchor it to the stroma. Considering also that their integrity is ensured by a heterogeneous class of molecules (melatonin, procyanidins, epigallocatechin etc.), one may, in the context of countermeasures, preliminarily test such substances, in order to assess the extent to counteract the effects of the reduction of gravity.

ACKNOWLEDGEMENTS:

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NANOPLASMONICS ISSUES FOR IMPROVING CANCER TREATMENTS

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Keywords: Nanoplasmonics, gold nanoshells, Near-Infrared transparency windows, Scanning Near Field Microscopy

INTRODUCTION

Photothermal therapy (PPT) as well as photodynamic therapy (PDT) are cancer treatments that use light in combination with photosensitizing agents or drugs to kill cancer cells. These techniques can be greatly improved if adequately developed within the new paradigms that are emerging from theranostic nanomedicine.

Because of their strongly resonant light-absorbing and light-scattering properties that depend on shape, noble metal nanoparticles (NPs) provide a new and powerful tool for innovative light-based approaches, in particular gold NPs, AuNPs. At wavelengths just beyond the visible spectrum in the near-infrared, blood and tissue are maximally transmissive. As a consequence, by manipulating AuNPs shape and dimension, it is possible to tune the optical resonance of such NPs to this region of the spectrum so that they become useful contrast agents in the diagnostics imaging of tumors. When illuminated by an external near-infrared (NIR) source, NPs can serve as nanoscale heat sources, photothermally inducing cell death and tumor remission [1]. The use of plasmonic nanoparticles as highly enhanced photoabsorbing agents has thus introduced a much more selective and efficient cancer therapy strategy, viz. plasmonic photothermal therapy (PPTT). The synthetic tunability of the photothermal properties and the biotargeting abilities of the plasmonic gold nanostructures make the PPTT method furthermore promising.

PPT and PDT can work analogously to surgery or radiation therapy, in treating certain kinds of cancers and pre-cancers, figure 1.

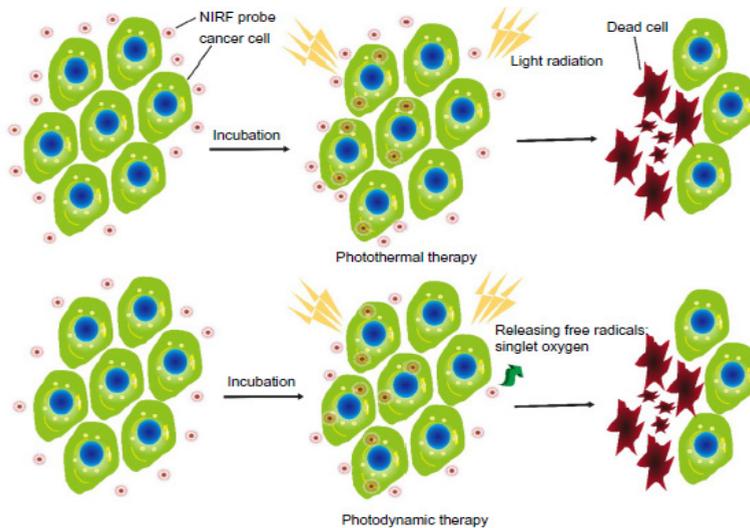


Figure 1: Schematic illustration of PPT and PDT. The Near-Infrared probes, in our case, the AuNP eventually conjugated with dyes, peptides, antibodies, etc. are incubated in the cancer cells. Upon light radiation, the accumulation of AuNP probes drastically increases the efficiency of PPT through the effective conversion of light energy into heat, resulting in a laser-induced thermal damage to cancer cells. In PDT settings, the AuNP probes facilitate the generation of cytotoxicity-free radicals as singlet oxygens, and initiate an inflammatory microenvironment that leads to cancer cell death after light radiation.

Many of the critical aspects connected to PPT and PDT can be dropped working with light falling in the near-infrared (NIR) region ranging from 750 to 1100 nanometers, where the majority of cells show the so-called NIR transparency window, where either absorption or scattering of light are strongly minimized and tissue penetration depths of some centimeters can be achieved [2].

RESULTS AND DISCUSSION

The interaction between single cell- single AuNP should define and delineate the basic key-mechanisms induced by the conjugated biomolecules for selective cellular uptake for cancer cells. The best conjugating biomolecules with AuNP able to recognize and bind target cells. In turn, their response to illuminating NIR light when internalized in

the cancer cells will be studied. The response of optical properties will be also studied illuminating the cells with a time-resolved ultrafast laser.

This innovative contribution is based on the recent progress made by D'Acunto and co-workers demonstrating the possibility to identify single AuNPs with dimensions 100-150nm, inside a single h9c2 mouse cell, figure 2, using an illumination wavelength of 780nm. This demonstration opens the possibility to study the single NP uptake and the inside cell thermal induced effect before the cells being eventually destroyed.

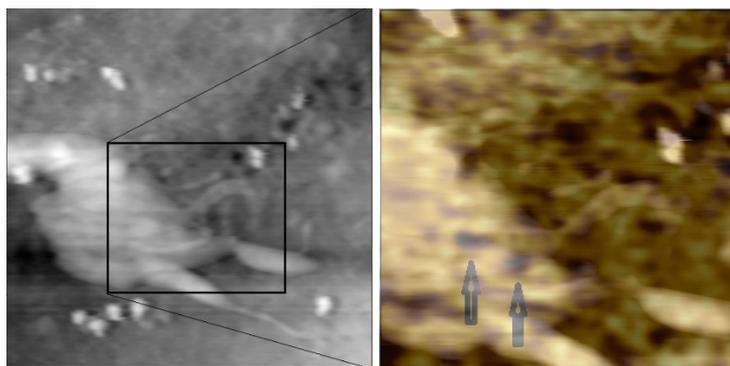


Figure 2: (Left), $20\mu\text{m}\times 20\mu\text{m}$ topography of h9c2 mouse cell recorded with a home-made aperture SNOM; (middle) an overlapping of the topography image with the correspondent optical map, the arrows denote the (120nm diameter) NP identified within the cell; (right), strong-absorption points marked by arrows in the overlapping image and denoting the dimensions of the NSs. (Source: D'Acunto *et al.*, [3])

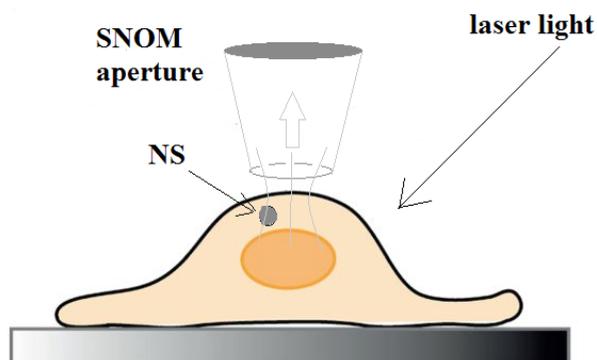


Figure 3: Schematic sketch representing the cell+NS (in this case NS denotes a nanoshell, a particle made by a dielectric core and a gold shell of 20nm layer) sample with aperture SNOM probe.

Such progress and the correspondent ability to study the interaction between single AuNP- single cell has been made possible by the usage of Scanning Probe Microscopy (SPM). SPM denotes a versatile family of scanning microscopies with spatial resolution of nanometers. Measurements could be made with a combined microscope reaching the basic features of Atomic Force Microscopy, AFM, and scattering or aperture Near Optical Microscope, SNOM. Such microscope, can work in a wide variety of environments, in air, as well in liquid, at physiological conditions, and with a wide range of light wavelengths.

The basic goal is to study the basic interaction between single AuNPs and single cancer cells, in the NIR region of light, opening the possibility to observe directly the thermal effects induced by the NP on the cell components and organs.

One critical problem is the determination of the so-called *z-localization* of the AuNPs inside the cells. This basic question was addressed providing a numerical demonstration that the SNOM is able to locate AuNPs inside the cells with a particle-aperture distance of about 100nm. This result was obtained developing an analytical approach based on the calculation of the dyadic Green function in near-field approximation. The implications of our findings will remarkably affect further investigations on the interaction between NPs and biological systems [4]. A correspondent experimental demonstration of such *z-localization* is one of the next tasks of our work.

CONCLUSIONS AND OUTLOOK

The use of plasmonic nanoparticles as highly enhanced photoabsorbing agents has thus introduced a much more selective and efficient cancer therapy strategy, the plasmonic photothermal therapy. The synthetic tunability of the photothermal properties and the biotargeting abilities of the plasmonic gold nanostructures make the plasmonic photothermal therapy method furthermore promising. In this paper, we have presented how noble metal nanoparticles (NPs) provide a new and powerful tool for innovative light-based approaches, in particular gold NPs, AuNPs, due of their strongly resonant light-absorbing and light-scattering properties that depend on shape. At wavelengths just beyond the visible spectrum in the near-infrared, blood and tissue are maximally transmissive. As a consequence, by manipulating AuNPs shape and dimension, it is possible tune the optical resonance of such NPs to this region of the spectrum so that they become useful contrast agents in the diagnostics imaging of tumors. When illuminated by an external near-infrared (NIR) source, NPs can serve as nanoscale heat sources, photothermally inducing cell death and tumor remission.

The combination of Atomic Force Microscope and Scanning Near-Optical Microscope can help the detection and identification of AuNPs inside the cells. One critical problem inherent such detection is the z-localization of AuNPs inside the cells. This problem has been addressed providing a numerical study. The results show that the AuNPs can be detected inside a deep penetration distance of almost 100nm.

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RAMAN IMAGING OF TISSUES TOWARDS CLINICAL CANCER DIAGNOSTICS

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Keywords: Raman spectroscopy, imaging, diagnostics, cancer, tissues, fingerprint

INTRODUCTION

Raman spectroscopy (RS) is an optical high specificity label-free method increasingly applied to get molecular fingerprints of biological tissues and cells. Recent technological advances have created a novel and fast Raman imaging microscope instrument, providing morphological investigation of large tissue areas, coupled with point-by-point spectral analysis of biochemical composition. This modality is important not only for discrimination between healthy and pathological tissues, but especially for gaining specific molecular information on the gradual biochemical changes from a healthy tissue to a tumor state and for the identification of key components responsible for tumorigenesis and its leading events.

The aim of the present work is to demonstrate that RS is able to reliably discriminate between healthy and papillary thyroid carcinoma (PTC) tissue and is suitable for the improved non-invasive diagnosis and detection of thyroid tissues. Our results demonstrate that the achieved diagnostic accuracy is compatible with the clinical use and that RS imaging of human tissues may improve histopathological evaluation.

RAMAN SPECTROSCOPIC MEASUREMENTS

Raman spectra were recorded using a Thermo Fisher Scientific DXRxi Raman microscope (RM) at the following conditions: 532nm laser source; 200-3400 cm⁻¹

full range grating; 10x and 50x objectives; 25 μm confocal pinhole, 5 (FWHM) cm^{-1} spectral resolution. The indicated above RM instrument guarantees a fast change of experimental parameters, for better measurements procedure optimization. Laser spot size was about 700 nm (50x objective). Various Raman maps of tissue zone size from $100 \times 100 \mu\text{m}^2$ up to $2 \times 2 \text{mm}^2$, collecting several thousands of spectra per map, were obtained. For each tissue section several Raman maps containing up to 30 000 spectra per map were analysed.

STATISTICAL ANALYSIS

Statistical analysis was performed on average spectra of each map, corresponding to the 30 healthy tissue average spectra and the 30 PTC average spectra. The FP and HWN range of Raman spectra, roughly $600 \div 1800 \text{cm}^{-1}$ and $2800 \div 3100 \text{cm}^{-1}$, respectively, were selected for statistical data analysis treatment. The remaining spectral range was not considered, as non meaningful from the point of view of the contained biochemical information. The collected Raman data were processed performing multivariate analysis, used for complex systems with high internal variability. Two principal statistical procedures were performed on the dataset: Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA).

At first, PCA was carried out in order to reduce the initial high dimensionality of the dataset and to verify whether, in a subset of dimensions, the variability of the different tissue typologies can reveal on its own differences among samples that could be considered diagnostic. LDA was applied on the same components observed in PCA to verify if differences among the typologies do exist due to differences among the means of samples belonging to different tissue groups. Moreover, the implementation of the algorithm on principal components (PCs) let to optimize the process of classification on the basis of a reduced number of input variables. The LDA was controlled by the leave-one-out cross validation method.

DATASET

A number of thyroid tissue areas from 10 patients were histologically identified and diagnosed as healthy or pathological (PTC) (Haematoxylin/Eosin staining of frozen samples) by an experienced pathologist (A.C.). By means of the RS imaging, 30 maps have been obtained from healthy and 30 from pathological areas.

RESULTS AND DISCUSSION

A great interest nowadays is focused on the biochemical profile of tumours in view of availability of new drugs that specifically target neoplastic cells. This new paradigm requires biochemical analysis of each tumour in order to establish the correct personalized oncological “target therapy”. Understanding the mechanism of molecular alterations of a tumour is a critical issue to prognosticate its behaviour and to predict the response to a specific therapy.

There are many recent studies strongly suggesting that RS can be used as a clinical tool for cancer diagnosis, improving accuracy in decision-making [1-3]. In vivo RS clinical applications are extremely challenging and nowadays remain to be applied only in a few countries [4-6]. These studies are limited by the time-consuming spectral measurements, throughout accepted standard data treatment procedures and by the absence up to now a Raman human organs databases. However, recent advances in optical technologies and instrumentation, rapidly growing clinical translational purpose RS investigations, and a European COST action network created in order to coordinate the research groups will certainly lead to a breakthrough and the spread of in vivo applications of RS in clinics [4-6]. With this regard, the aim of the present research is to demonstrate that Raman spectroscopy with the increased acquisition speed is able to reliably discriminate between healthy and carcinoma tissues, based on its biochemical profile.

Furthermore, RS has the ability to identify specific tumour expression molecules and molecular species involved in tumourigenesis and progression. Here we show the typical RS biochemical maps taken on the cryo-sectioned Thyroid tissues.

In Figure 1, a Raman map of the healthy Thyroid tissue is shown. Each colour corresponds to a certain kind of spectrum, and hence to a certain chemical composition.

In Figure 2, a Raman map of the pathological Thyroid tissue is shown. The pathology corresponds to the Papillary Thyroid Carcinoma. Each false colour corresponds to a certain kind of spectrum, and hence to a certain chemical composition.

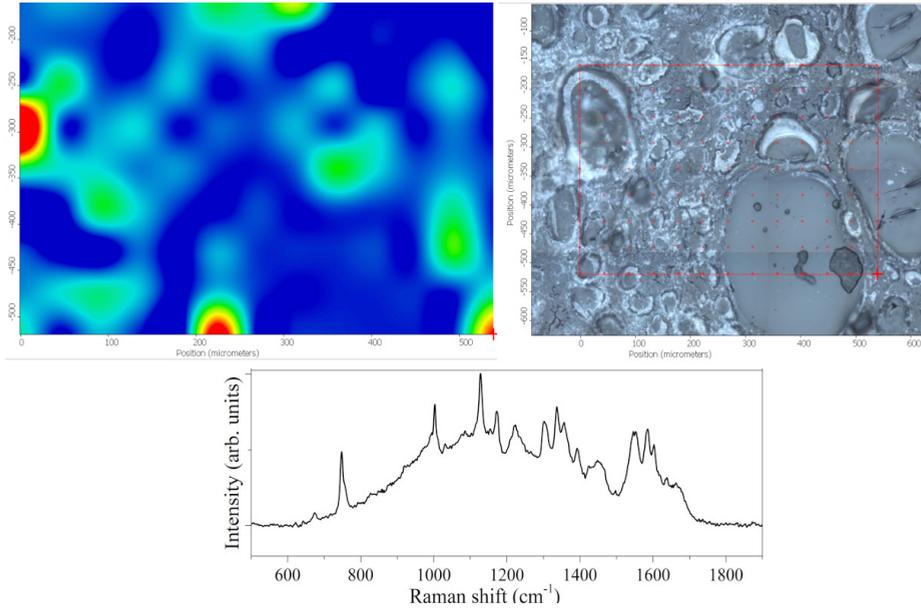


Figure 1: Raman map of the healthy Thyroid tissue.

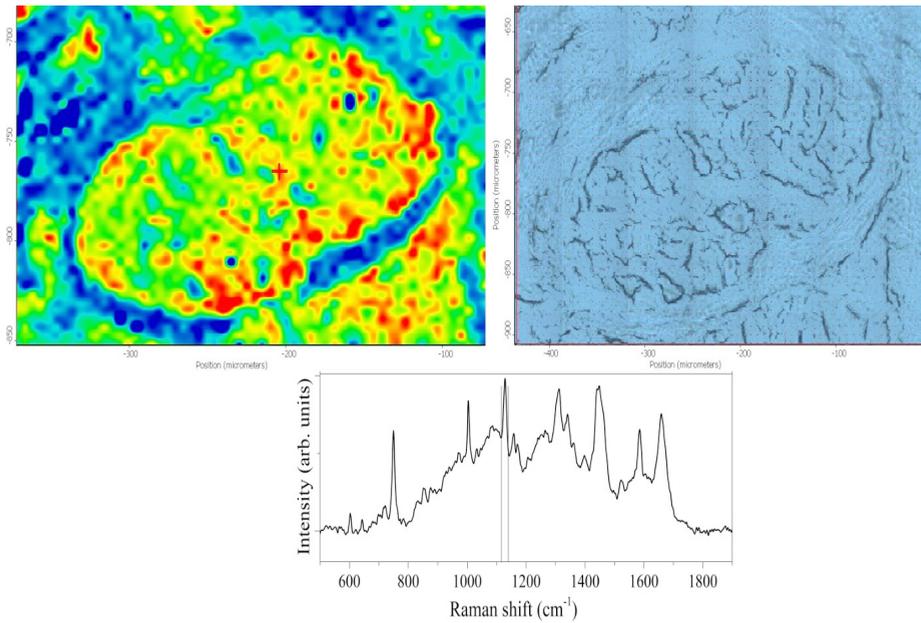


Figure 2: Raman map of the pathologic Thyroid tissue. Pathology corresponds to the Papillary Thyroid Carcinoma.

CONCLUSIONS AND PERSPECTIVES

RS is an important tool for tissue imaging and early cancer diagnostics. It is expected to produce a major advance in imaging of malignant tissues, leading to the development of portable diagnostic devices for hospital use for various types of cancer. It is also planned to utilise the powerful combination of high spatial resolution and chemical specificity of the mentioned methodologies to study the key components, responsible for cancer formation.

The combined histological and Raman microscopy analyses allow integrated morphological and biochemical observations, with the significant improvement of efficiency and reliability of the differential diagnosis of neoplastic thyroid nodules, paving the way to integrative findings for tumorigenesis and novel therapeutic approaches.

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NON-DESTRUCTIVE MEASUREMENT OF PH IN TISSUE ENGINEERED SKIN USING CONFOCAL RAMAN SPECTROSCOPY.

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Keywords: pH, skin, tissue engineering

INTRODUCTION

Skin is designed so that the upper layer provides an acid barrier mantle of around pH 5.5. Beneath the upper barrier layers of skin the pH rises to around 7.5 to 7.8. One of the big challenges of wound healing research is to investigate what role, if any, pH plays in wound healing. Crucially wounds can be inflamed or bacterially infected and both conditions which retard healing may change the pH environment. The challenge is that it is technically difficult to measure pH at different points within skin in a non-destructive manner. For solutions of liquids pH electrodes are the method of choice. For monolayers of cultured cells fluorescent ion sensitive dyes can be used but in our experience neither work well in 3D tissue. Currently it is difficult to obtain meaningful pH measurements in man or animals. Accordingly, we have used a 3D human skin model in which to assess the use of confocal Raman spectroscopy for measuring pH in control, wounded and inflamed skin conditions.

RESULTS AND DISCUSSION

Tissue engineered models of normal skin (TE skin) were created from human acellular dermis seeded with normal human keratinocytes and dermal fibroblasts [1] and cultured at an air liquid interface for 14 days. For models of inflammatory skin, models were treated with 20ng/ml Il-17 for 7 days prior to measurement.

Epithelial wounds were created by selectively masking a region of dermis and removing this masking for 7 days prior to measurement.

Raman spectroscopy measures the Raman shift of light after it has interacted with molecular vibrations giving information about the vibrational state of the target. The technique can give fingerprints which show the chemical state of compounds. Combining such spectroscopy with a confocal imaging system allows us to obtain information from the surface and interior of a sample in a non-destructive and non-contact method. By monitoring chemical species which are sensitive to pH in a predictable and 'simple' fashion we can deduce the pH. Inorganic phosphates and terminal phosphate groups show pH induced changes in protonation between pH 8 and 5.5, their pKa is approximately 7. Vibration of the base form PO_3^{2-} gives a strong signal peak at 980cm^{-1} and the acidic PO^{2-} gives a signal at 1082 cm^{-1} . As pH changes from 5.5 to pH 8 there is a symmetrical increase in the 980 peak and a fall in the 1082 peak. A ratio of the peak intensities can be used in the Henderson Hasselbalch equation - $\text{pH} = \text{pKa} + \log_{10} (\text{base/acid})$ or $\text{pH} = \text{pKa} + \log_{10} (\text{Intensity}_{980} / \text{Intensity}_{1082})$.

Raman spectra were taken at $15\ \mu\text{m}$ intervals across $150\ \mu\text{m}$ of the epidermis and at intervals of $50\ \mu\text{m}$ deep down to $400\ \mu\text{m}$ into the epidermis of tissue engineered skin in PBS at pH 7.3-7.4. The upper surface was dabbed dry of PBS, and spectra obtained with a 720nm laser which resulted in spectra with peaks at Raman shift of 1074cm^{-1} , and 986cm^{-1} approx. [2] Figure 1 shows pH data from tissue engineered models and Hematoxylin & Eosin stained histological sections of each model.

In control TE skin (Figure 1A), from the top surface of the skin, the pH is 7.4, this then changes to an acidic pH 4.75 by $150\text{-}200\ \mu\text{m}$ into the TE skin, and then at greater depths the pH then sharply returns to pH 7.4-7.5. Treating TE skin models with inflammatory interleukin IL-17 creates the disrupted epithelium seen in some inflammatory skin conditions. Previous unpublished work using IL-17 and IL-22 showed that treatment with either can induce an abnormal epithelial morphology resembling psoriasis with disordered differentiation, nucleated cells appearing then the upper granulosum and corneum, and an overall fragile looking epithelium.

The pH of this damaged epithelium (B) remained between pH 6.25 and 7.5, the acidification of normal epithelium was missing (Figure 1B). Similarly, in the wounded TE skin where the epithelium was largely absent, the pH varied between 6.5 and 7.5 (Figure 1C).

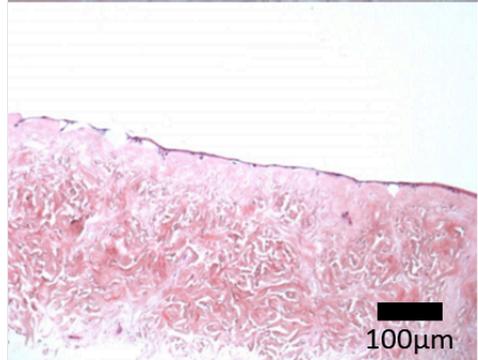
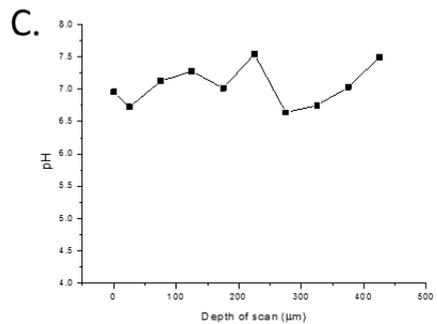
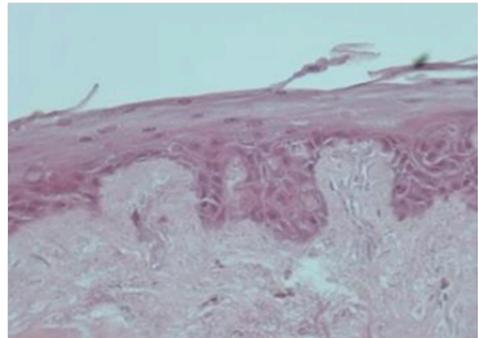
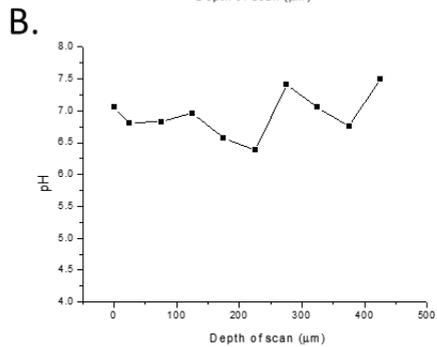
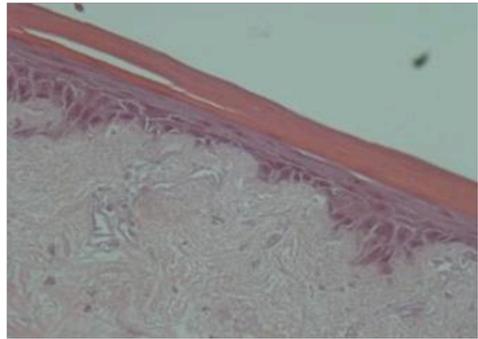
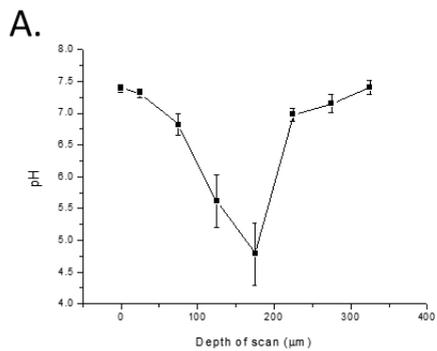


Figure 1: pH data from TEskin models and H&E stained sections of TEskin skin. A) control TEskin, B) inflamed TE skin and C) wounded TEskin.

CONCLUSIONS AND/OR OUTLOOK

Our measurements show the acidification of the upper epidermal layers (acid mantle) reaches much deeper into the epidermis than in vivo measurements gained from tape stripping/application and use of a flat pH electrode.[3] We also found that damaging

the integrity of TE skin either with IL-17 (20ng/ml for 7 days) or creating an epithelial wound led to loss of the acid pH environment seen in normal TE skin. pH values from the start of the scan/measurements indicate a pH of 7 for the upper surface, and this may be measurements of PBS remaining on/in the upper layers of the corneum, but these values are similar to those seen in ex vivo and frozen skin.[3] Where we have induced inflammation, the absence of the acidic region in the epidermis shows that the treatment has upset the normal process of differentiation, preventing the creation of the acidic environment. This model more resembles psoriasis, we have previously published on a tissue engineered model with similar architecture[4][5] where there is a hyperproliferation of basal cells which leads to a derangement of the epithelial morphology. sPLA₂ has been seen to become localized to the spinosum and epidermal region rather than the corneum: granulosum border – leading to perhaps a gross change in the production of free fatty acids and hence a poor acidification barrier.[5] The histology confirms that disruption /malformation and loss of the corneum due to IL-17 treatment reduces the acidic region of the epidermis. In conclusion we report a non-invasive method of measuring pH at varying depths in 3D skin which will allow us to study the natural pH gradient in skin in healthy skin and in models of inflamed and wounded skin. This also has the potential to be developed for clinical use.

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WEARABLE MICROPROJECTION ARRAY SKIN PATCHES FOR SAMPLING BIOMARKERS FROM THE SKIN

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Keywords: microneedles, skin, biomarkers, wearable diagnostics, surface modification

INTRODUCTION

We have demonstrated Microprojection array skin patches (MPAs) capture circulating blood biomarkers from the skin as a needle-free alternative to traditional blood sampling [1-3]. Currently, most clinical blood based biomarker assays rely on sampling blood via a needle and syringe. The blood is then processed at centralised laboratories to perform diagnostic assays in vitro. This approach limits the frequency of diagnostic tests and is not suitable for continuous monitoring. The skin, due to its abundance of superficial capillary vessels (Figure 1a), offers an alternative route to collect circulating biomarkers with minimal invasiveness for more frequent or continuous monitoring. This diagnostic potential, however, has been largely unrealised due to the lack of standardised and convenient methods to sample biomarkers from the skin. To address this challenge we engineered microprojection arrays to penetrate only the upper layers of the skin (Figure 1b). We then surface modified these projections with capture probes to bind circulating disease markers from skin fluid, while MPAs are worn on the skin. These capture probes selectively bind circulating proteins by affinity interactions in vivo, thus avoiding the bulk sampling of fluid. We have demonstrated MPAs capture dengue, malaria and IgG (antigen specific) from the skin, in animal models. The device can then be extracted for use with in vitro diagnostic assays or potentially integrated with portable biosensors.

Accessing the biomarker of interest, however, from the complex milieu of the tissue environment remains a key challenge critical to enable high MPA detection sensitivity.

This requires optimisation of the interface (surface area and chemical functionality) between the MPA and the tissue. Effects of degradative or fouling species from the tissue on the functionality of the capture surface *in vivo* require investigation to develop lower fouling and more selective capture surfaces which remain functional *in vivo* over extended periods. Furthermore, the biocompatibility and tissue response to MPA application may result in a local changes, extravasation and inflammation, that affect sampling whilst MPAs are applied to the skin.

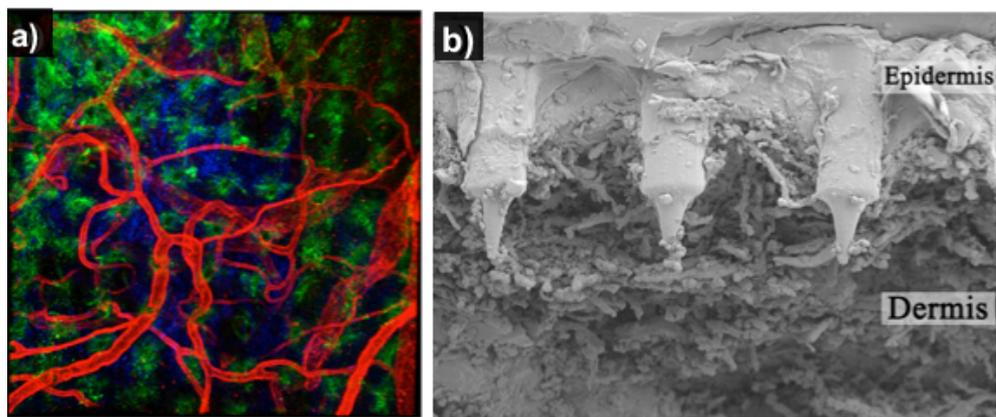


Figure 1: a) Blood vessels in murine skin (red) overlaid with MPA penetration (in green) to these vessels b) Cryogenic scanning electron microscopy micrograph of MPA penetrating skin tissue. Epidermal ($\sim 20\mu\text{m}$ thick) and dermal ($\sim 160\mu\text{m}$ thick) skin layers are indicated

RESULTS AND DISCUSSION

We report the use of microprojection arrays to sample IgG, dengue (NS1) and malaria (HRP11) disease markers with minimal invasiveness from the skin of mice. We then characterise the effect of microprojection array design (length, density, array size) on biomarker capture and demonstrate that biomarker capture increases with the tissue contact surface area of the MPAs. The effect of MPA application and projection design on plasma protein extravasation from skin vasculature and the concentration/turnover of biomarkers in skin was also investigated. We found that MPA insertion induces plasma protein extravasation and increases biomarker concentration, which may play a key role in both accessing circulating blood biomarkers and synergistically

increasing their capture onto microprojections in vivo [4]. Complementary approaches to induce extravasation, separate to MPA application, enabled further increase MPA biomarker capture. Combining this with our optimal MPA design we demonstrate MPAs rapidly, reliably and reproducibly sample biomarkers (antigen specific IgG) for immunoassays in less than 1 min – currently the best reported sampling time and diagnostic sensitivity using microprojections, making them highly suitable for rapid diagnostic tests.

The functionality of the capture surface in vivo was also investigated over longer sampling times relevant for continuous monitoring. There was a significant decrease (~60 %) in specific biomarker capture and increase in non-specific background signal to explanted MPAs over 24 h, suggesting significant degradation or fouling of the capture surface reducing its functionality. An inflammatory response (influx of neutrophils) was also observed in the tissue surrounding the MPA, which may contribute to this surface degradation. Preliminary studies with zwitterionic antifouling polymer coatings (polysulfobetainemethacrylate) show improvement in biomarker capture over shorter sampling times (<10 min), which may offer promise to improve long term sampling. A key remaining challenge is to identify the causes of this functionality loss and to develop stable surfaces for long term in vivo sampling.

CONCLUSIONS AND/OR OUTLOOK

MPAs show great promise as rapid sampling devices to selectively extract circulating biomarkers from the skin, without requiring sample purification and with minimal invasiveness. This makes them suitable for use with rapid and portable diagnostic tests. We also envisage MPAs may be used perform long term measurements, or for continuous monitoring, over longer application times as a wearable device. Key remaining challenges such as developing a stable capture surface that resists degradation, fouling and the effects inflammation in vivo, whilst freely allowing proteins in the skin access to surface, require investigation.

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EFFICIENT DELIVERY OF MIRNA BY POLYAMINE-COATED CARBON NANOTUBES AS INNOVATIVE THERAPEUTIC AND DIAGNOSTIC TOOLS

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Keywords: miRNA, multifunctional carbon nanotubes, polyamine

We used pristine and carboxy (COOH) functionalized carbon nanotubes (CNTs), obtained following well-established methodologies [1], for preparing functional nanomaterials coated with polyethylenimine (PEI) and polyamidoamine (PAMAM) polymers. The latter have been employed to obtain drug delivery vectors for in vitro and in vivo biomedical applications [2,3]. Simple physicochemical adsorption allowed us to coat CNTs, whereas polymer-conjugated CNTs have been obtained by amide bonds formation by taking advantage of COOH functionalities. Different functionalized CNTs display different solubility and toxicity properties, as a function of the polymer molecular weight. We evaluated the properties of these functional nanomaterials (i.e. DNA binding ability, toxicity, transfection efficiency) by specific in vitro assays (i.e., gel electrophoresis, cell cultures and fluorescence microscopy) on human primary endothelial cells. Our results illustrate the versatility of such innovative devices as multifunctional vectors for in vitro delivery of DNA/RNA-based oligonucleotides. This work is supported in part by the Italian Ministry of Health research project "Delivery and imaging of miRNAs by multifunctional carbon nanotubes and circulating miRNAs as innovative therapeutic and diagnostic tools for paediatric pulmonary hypertension" (PE-2011-02347026 – Years: 2014-2017).

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CYTOTOXICITY OF DIFFERENT SERS-ACTIVE NANOVECTORS BASED ON ANTI-FOLATE DRUGS

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Keywords: Cytotoxicity, SERS-active nanovectors, anti-folate drugs, theranostics.

INTRODUCTION

Nowadays, early diagnosis of cancer is one of the main biomedical purposes and its achievement involves several scientific disciplines such as physics, chemistry, biology and nanotechnology. Over the last few years, several methods have been tested and developed from standard assays to really innovative techniques for selective bio-recognition of cancer cells. Surface Enhanced Raman Spectroscopy (SERS) is one of the most exploited methodologies in this respect, and it involves the realization of nanostructured bioplasmonic vectors[1,2]. A SERS- active nanobiovector consists in a metallic nanoparticle (Np) core functionalized with a suitable Raman active biomolecular layer. The optical (visible/IR) excitation of the electron cloud of the metal nanostructure produces the localization of extremely intense electromagnetic fields at the metal surface [3]. This phenomenon allows for revealing the Raman signal of the molecules located near the metal surface, thus overcoming the limits of Raman sensitivity. The advantages of SERS in biomaterials investigations are the possibility of tracing Nps across a sample, to produce imaging and, if necessary, to perform photothermal therapy (PTT). In order to provide for selectivity in molecular and cell targeting, SERS nanovectors can also be functionalized with antibodies or other small ligands able to recognize

specific molecular receptors (e.g. membrane proteins over-expressed in tumour cells) [2,4]. In our recent work, we proved high sensitivity and selectivity of folate-conjugated Nps, moreover adding theranostic features by substituting folate with anti-folate drug Aminopterin (AMT) [2]. Herein we present the characterization of these nano-biovectors, enlightening in particular their level of cytotoxicity on human cancer cells (HeLa) compared to a immortalized cell model (HaCaT). We discuss the tremendous therapeutic potential of our novel anti-folate conjugated SERS-nanovectors.

RESULTS AND DISCUSSION

To achieve the early diagnosis of cancer we propose high sensitive and selective nanovectors. The high sensitivity is realized by gold Np that show a plasmonic response if illuminated with visible/IR light. The selectivity, otherwise, is carried out by the molecular functionalization of the Np surface with folic acid. This molecule is known to be an important nutrient for cellular growth and for this reason cancer cells overexpress on their membrane folate receptors. First of all we will show the correct functionalization of the Nps conjugated with folate or anti-folate performing UV-visible absorption, Z-potential and dynamic light scattering measurements and SERS characterizations. It is also possible to calculate the number of molecule on the surface of each Nps through a titration test.

Since we have investigated the spectral differences of the two nanovectors and their functionalization, we focused on cytotoxicity to prove the therapeutic effect of the Nps conjugated with anti-folate instead of folate.

AMT, like all anti-folate drugs, to act against cancer cells must be internalized so that its cytotoxic effect is triggered by the irreversible binding to the folate metabolic enzymes, preventing folate action in DNA and protein biosynthesis. The cytotoxicity was assayed by MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and the results have shown a higher cytotoxicity activity on HaCaT and HeLa treated with AMT-conjugated Nps compared to AMT free (Figure I.A). At this regard, the 50% inhibitory concentration (IC 50) of AMT-conjugated Nps was significantly lower for both cell lines, primarily for HeLa cell line (Figure I.B). These results suggest as functionalized SERS-active Nps may represent an important method for selective bio-recognition of cancer cells as well as an effective drug delivery system.

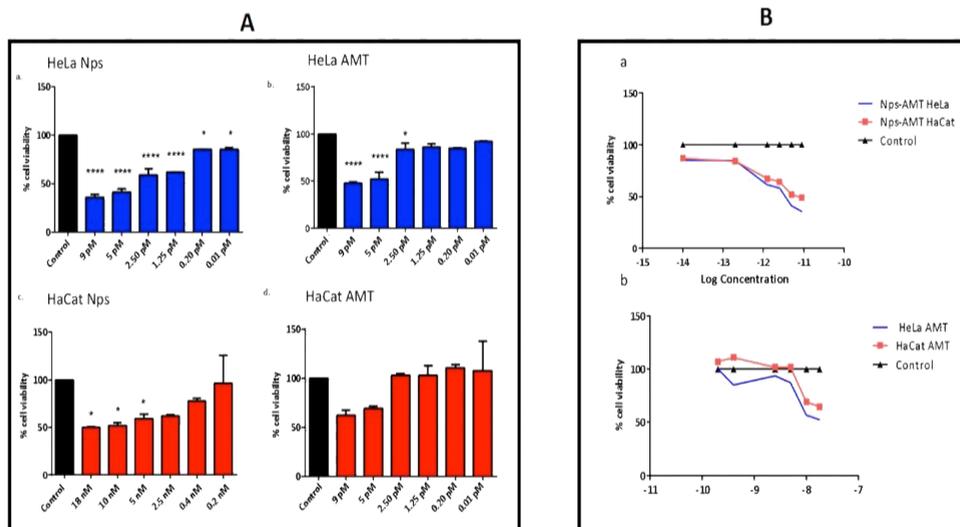


Figure 1: A) MTT assay showing the cell viability of HeLa (a-b) and HaCat cell lines (c-d) treated with different concentration of Nps-AMT and AMT free for 24 hrs. **** p \leq 0.0001, *** p \leq 0.001, ** p \leq 0.01, * p \leq 0.05 (the significance was evaluated by the Tukey honestly significant difference (HSD) post hoc test); B) Cell viability showing an IC50 value equal to 3.23 pM and 4.43 pM in HeLa and HaCat cell lines treated with Nps-AMT (a) and 18.71 nM and 36.91 nM with AMT treatment (b).

CONCLUSIONS AND/OR OUTLOOK

In this work we proved, after a deeply characterization of our nanovector, the high cytotoxicity of AMT-conjugated Nps against the absence of mortality in folate-conjugated ones in HeLa cells. The graph (Figure 1.A) shows that at the same concentration the cytotoxic effect is significantly higher when treated with Nps-AMT with respect to AMT free.

Further investigations based on the design of several Nvs with a co-capping of folate are ongoing to improve the selectivity among different cell lines expressing folate receptors and carriers, and AMT to maintain high cytotoxicity.

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REDOX-SWITCHABLE OF POLYDOPAMINE ON TITANIUM SURFACE FOR REVERSIBLY REGULATION OF CELL ADHESION AND PROLIFERATION

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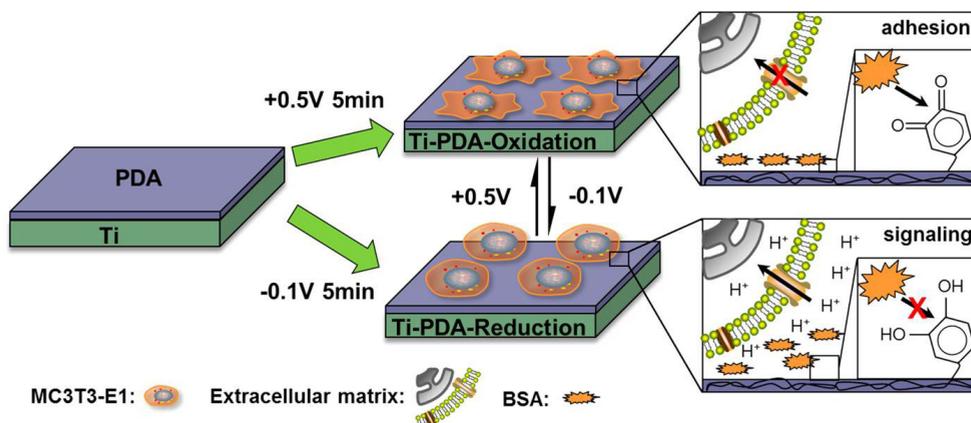
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Keywords: Electrical Stimulation; Redox-Switchable; Polydopamine; Titanium; Cell adhesion

INTRODUCTION

Titanium implants, represented by biomedical metal implants, are well known for bone implant applications but insufficient integration into the surrounding bone often occurs for bioinert properties. The bioinert surface of titanium, in particular non-variable surface, cannot induce interactions osteoblast cells via adsorption of proteins in conjunction with the interface of host-biomaterial [1]. Interestingly, host-biomaterial interactions were tuned by surface chemistry or topography, which dictate the biological response in close proximity with the bone tissue. It was being rapidly developed a “controllable surface” for modulating the host-biomaterial interactions, such as the adhesion, spreading, proliferation and differentiation on the titanium implants [2]. Therefore, tuning surface chemistry, like surface charge or chemical constitution, has been proposed to control their biological activities from non-variable surface of titanium. Ideally, an implant should be “intelligent” so that it is able to reversibly change different surface properties through reversible transformation of molecule. Herein we demonstrate the first-time use of a bio-adhesion molecule, polydopamine (PDA) as such a redox molecule of dopamine/dopaminequinone

and leucodopaminechrome/dopaminechrome^[3], to achieve the reversible switch of surface properties in response to a potential switch (Scheme 1) as well as cell adhesion and proliferation on the titanium.



Scheme 1: Mechanical illustration of redox-switchable on Ti-PDA. The transformation of phenolic/quinone groups can depend on redox of Ti-PDA by applied potential, and the quinone groups upregulate BSA adsorption and promote cell spreading. Additionally, the hydrogen ions of phenolic groups on PDA have signalling on cell to differentiation via the osteogenic pathway.

RESULTS AND DISCUSSION

The stimuli-responsive thin films have been accepted widely in tissue engineering to further increase bone-implant osseointegration. The redox-switchable of polydopamine have been compared with the responses obtained from the electrical stimulation. In the present study, it was demonstrated that the redox process of polydopamine was a one-proton (H^+) coupled two-electron process ($2e^-$). The redox-switchable reversible surface potential, arising from the potential-tunable redox reaction of the phenolic and quinone groups of PDA, led to redox-switchable adhesion and spreading. In vitro experiments demonstrate that quinone groups of polydopamine at +0.5V can greatly enhance the cell spreading and proliferation of pre-osteoblasts (MC3T3-E1), and phenolic groups at -0.1V were conducive to induce differentiation. As depicted in Figure 1e-1h, MC3T3-E1 cell spreading area on Ti-PDA-Oxidation was larger than that on pTi and Ti-PDA-Reduction after 4 h adhesion. Ongoing experiments are examining the effect of osteogenic gene expression cues on cell morphology.

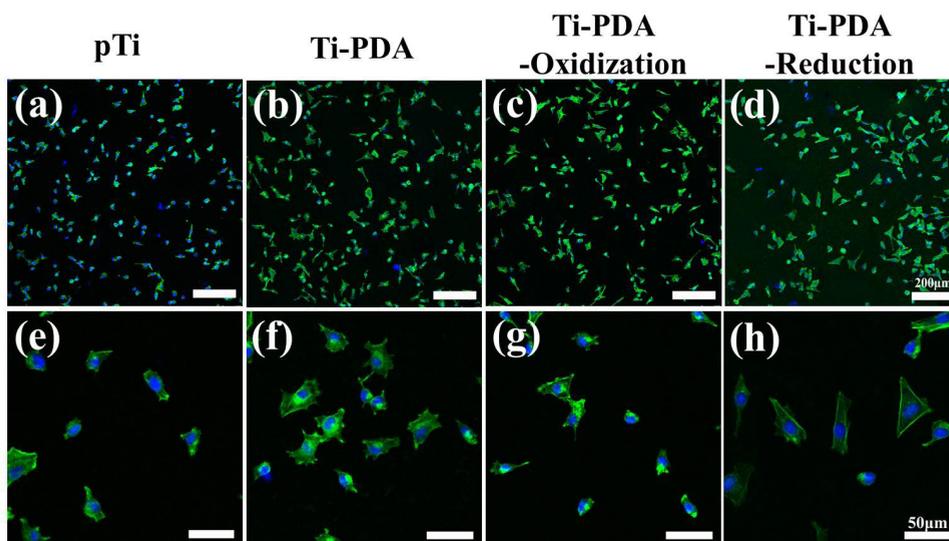


Figure 1: The cell spreading of redox-switchable in Ti-PDA. F-actin and DAPI staining for MC3T3-E1 on (a,e) pTi, (b,f) Ti-PDA, (c,g) Ti-PDA-Oxidization and (d,h) Ti-PDA-Reduction after being cultured for 4h. The cells density: 2×10^4 cells/mL. The cytoskeletons with extracellular proteins were spreading on Ti-PDA-Oxidization, yielding microfilaments of attachment. The adhesive properties of Ti-PDA were transformation from phenolic groups to quinone groups.

OUTLOOK

In this work, the phenolic/quinone transformation of PDA was achieved by electrochemical controlled for reversibly regulation of protein adsorption and cell growth. The reversible redox reaction of phenolic/quinone groups was controlled by electrochemical process enabling the PDA charge-transfer to change in surface potential and chemical composition. The phenolic/quinone transformation of PDA mediated anionic proteins accomplishes reversible regulation with applied electrochemical potential. The reversible redox reaction of phenolic/quinone groups enhances cellular viability and proliferation on Ti-PDA-Oxidization, and increased osteogenesis-related genes of MC3T3-E1 on Ti-PDA-Reduction. Owing to regulation of Ti-PDA by electrochemical, the phenolic/quinone groups of PDA can be extended to various types of biomedical materials that require osteoinductive properties. The proposed methodology may find applications in the area of monitor and regulation, especially for metal implant and potentially all biomedical devices with electroconductive surface as well.

ACKNOWLEDGMENTS

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STRUCTURAL AND PHASED STATE OF THE LOW MODULUS Ti-(40-45)Nb ALLOY FOR MEDICAL APPLICATIONS

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Keywords: Ti-Nb alloy, selective laser melting, structure, phase state.

Bioinert low-modulus alloys based on the Ti-Nb system have a special place among the alloys for medical applications [1]. The complex of their physical and mechanical characteristics, first of all the low modulus of elasticity, and bioinert properties, determine their application as materials for medical implants [1, 2]. The use of additive technologies, for example, the selective laser melting (SLM) method in the 3D printing mode, is in many cases the only alternative to the conventional methods of producing parts and items having complex shapes by molding. The development of the SLM method is especially perspective in implants and endoprosthesis production. Furthermore, this method allows producing items and parts with preset porosity, which is rather important for better osteointegration of the implant material with the bone tissue. The development of the SLM method and study of the structure and phase formation processes are important tasks. Features of structure and phase composition formation of the low elastic modulus Ti-40 wt.%Nb alloy obtained by SLM are investigated in the paper.

Composite powder of Ti and Nb was used to produce the Ti-Nb alloy by SLM method in the form of monolayer and multilayer samples with homogenous distribution of elements. Searching experiments to determine process conditions of synthesizing monolayer and multilayer from the Ti-Nb composite powder were carried out by experimental additive manufacturing facility «VARISKAF-100MB». This facility allows layer-by-layer SLM of powder products with diverse configuration. The composite Ti-Nb powder was produced using the mechanical activation method from pure

titanium and niobium powders in the planetary ball mill AGO-2.

Average content of Nb in the alloy varies within the range of 36–38 wt.%. The monolayer has a gradient composition with structure varying from fine-grained to medium-grained. Formation of the expressed gradient structure is associated with various temperature-time conditions of monolayer crystallization and cooling in the process of laser melting. Ti-Nb alloy has a two-phase state, namely the base phase of b-bcc-solid solution of Ti and Nb with grain sizes of 5–7 μm and non-equilibrium martensite a^2 -phase with grain sizes of 0.1–0.7 μm , locally distributed along the grain boundaries of the b-phase. The segregation of components is observed in the alloy. b-phase grains have an enhanced content of niobium, up to 45 wt.%, while observes a reduced content of niobium, up to 20 wt.% is observed in a^2 -phase grains.

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EVALUATION OF TWO AUGMENTATION MATERIALS OSSEOINTEGRATION IN SMALL DIAMETER BONE CAVITIES. AN IN VITRO LAB RATS STUDY.

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Keywords: bone graft, biomaterials, histomorphometry, Optical Coherence Tomography

INTRODUCTION

The use of grafting materials for socket augmentation might change the proportion of vital bone in comparison to sockets allowed to heal without grafting [1]. In general, autogenous bone is the most predictable material of choice for augmentation procedures, despite a 40% resorption, because it is highly osteoconductive [2]. However host morbidity limits autograft availability, so graft materials are likely to become more specialized for use in specific clinical applications, and composite preparations may soon provide bone graft materials with efficacy that equals or exceeds that of autogenous grafts [3]. The present study aimed to evaluate the degree of osseointegration of two synthetic bone augmentation materials.

MATERIALS AND METHODS

For this study we made three study groups, each of them consisting of six laboratory rats. On the calvaria and maxilla of these animals 3-mm diameter experimental cavities were carried out. For the first study group the cavities were augmented with the collagen fleece Alveoprotect (Bredent Medical, Senden, Germany), for the second group with the synthetic bone graft Ossceram nano (Bredent Medical, Senden, Germany), and in the third group the experimental cavities were left unaugmented.

After a healing period of two or four months, samples were obtained from the rats bones and subjected to three examination methods: clinical and radiological examination, Optical Coherence Tomography (OCT) and a histological study.

RESULTS AND DISCUSSION

In our study, in all the examined samples we noticed a good overall biocompatibility of the studied materials with the lack of any specific signs of inflammation. The cavities augmented with the collagen fleece Alveoprotect showed a faster bone healing comparing to the unaugmented cavities, both in maxilla and calvaria, the calvaria cavities filled with Alveoprotect being completely filled with bone tissue after 4 months. The cavities augmented with the synthetic bone graft Ossceram nano showed a quick formation of new bone tissue in the form of bone bridges, but we also noticed the presence of residual grafted bone particles, even in a significantly lower number in the cavities observed after 4 months of healing.

Optical Coherence Tomography (OCT) is a constantly growing imaging method for the medical investigation characterized by high spatial resolution and noninvasive subsurface detection [4]. Even penetration depth was limited approximately to 1 mm, the OCT allowed us to evaluate the surface and subsurface of the ongoing healing bone defects in a non-destructive manner.

For the in vitro methods the histological ones represents the classical evaluation of the bone graft materials biological integration. For the animal subjects, as we showed also in our study, stainless steel burrs are used to create 2-8mm in diameter and depth standardized intrabony defects in oral bones, but also could be made in calvaria or tibiae [5].

On the histological evaluation one may see the experimental defects filling and the new bone formation and also the intensity of inflammation, according to lymphocytic infiltration around the bone graft materials in the examined defects [6,7]. On the histological samples we generally noticed the experimental defects filling with connective tissue with various bone ingrowths from the surrounding bone tissue. The intensity of inflammation, according to lymphocytic infiltration may be assessed in the examined defects, as well as the presence of a foreign-body reaction, as manifested by the presence of foreign-body giant cells in a granulomatous response. Typical structures of a bone forming process were seen, with many blood vessels, thin bone containing numerous bone cells, a mineralization front with osteoid, and osteoblasts in a loose and cell rich connective tissue

CONCLUSIONS

Both studied materials proved a very good biocompatibility and provided faster bone healings. However, in the small bone defects it may be more advisable to use Alveoprotect in order to obtain a proper bone for implantation in a shorter period.

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A DEEP MORPHOLOGICAL CHARACTERIZATION OF NEW RESTORATIVE AND ORTHODONTIC MATERIALS: A COMPARATIVE STUDY

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Keywords: Invisalign, Giomer, Mechanical characterization, Raman spectroscopy, Ageing effects

INTRODUCTION

In dentistry, a new generation of restorative and orthodontic materials is continuously generating products and appliances with superior mechanical and esthetical properties. In particular, in orthodontics, thermoplastic polymers exhibit high elasticity and therefore low stiffness, good deformability, biocompatibility and dimensional stability, offering an optimum solution for a set of devices such as orthodontic retainers, temporomandibular joint splints, night guards and bleaching trays [1, 2]. Clear thermoplastic removable appliances have been introduced,

such as Invisalign® (Align Technology Inc., Santa Clara, CA, USA), which is made by CAD/CAM technology. In this way, it is possible to produce the kinds of movements required for comprehensive orthodontic treatment and, at the same time, to create more esthetic appeal than traditional stainless steel or esthetic brackets and wires [3, 4]. Invisalign® is mainly constituted by polyurethane that is a very promising thermoplastic polymer. This material, in fact, can ensure a high level of comfort for patients and this is the reason for its wide usage respect to traditional fixed orthodontic appliances.

Conversely, in restoration, giomer represents a new material with added benefits of high radiopacity, anti-plaque effect, fluoride release and recharge. Giomers are based on novel pre-reacted glass technology, in which specific fillers are included in the resin matrix [5].

In this work, we present a comparative study among different giomers and two types of orthodontic polymers with the intent of investigating the mechanical behaviour of these materials and their response to ageing effects in different fluids like human saliva, pbs and mild acid, usually present in commercial beverages. Raman, SEM and XRD analysis are also reported.

RESULTS AND DISCUSSION

Forty Invisalign® aligners were selected for experimental tests. They included 20 reference aligners (no intra-oral exposure) and 20 clinically used aligners worn intra-orally for two weeks, approximately 22 h each day. The samples consisted of 20 Smart Track aligners, including 10 never used (STN) and 10 used (STU), and 20 EX30 aligners, including 10 never used (EXN) and 10 used (EXU). In case of giomers, four different types of resin have been analyzed: Tetric (TE), Ketac (KE), Beatyful II (BE) and Dyract (DY). Several samples of each restorative material have been shaped in cylinders and tested.

To compare the mechanical properties of the different samples, they have been subjected to indentation tests. The hardness of the materials was calculated with a Vickers indenter as the deepness of the imprint caused by the Vickers tip over the samples. In figure 1a and b, we report the classical imprint for the two aligners and we summarize the hardness calculated for the different giomers.

In the imprint caused by the load during the indentation test, only the EX30 material showed a traditional residual imprinting, whereas the ST sample cracked, and its imprints were smaller, indicating, according to the results of the tensile strength

tests, that the ST material was more rigid than the EX30. In Table 1, the measured values are reported. In addition, to evaluate the mechanical properties after usage, some tests were performed on used samples of EX30 and ST materials. In this case, the indentation load (1 Kg) did not leave any imprinting on the samples, thus indicating that both materials were inclined to become harder with age and usage, changing their initial properties.

Ageing tests have been also performed, by analyzing their Raman spectra at 532 nm as is and after the immersion of the samples for 15 days in human saliva, pbs and a well-known sugary drink. With the aim of investigating the presence of biological traces on the used aligners, we collected a Raman spectrum of the used samples (STU and EXU). Comparing the spectra obtained on the surfaces of both samples, no differences caused by usage were observed in both materials, demonstrating the absence of significant contaminants on the aligners after usage. Conversely, in case of giomers, the surface of the samples reveals the presence of organic residues and sometimes the exposure to the mild acid solution slightly changes the surface of the resin. In fig.2a we report the Raman spectra for all the giomers before the ageing tests, while in fig.2b, which refers to BE, large and intense bands appear, ascribable to fluorescence signal associated with the presence of proteins after the immersion in saliva. These data are confirmed also after a prolonged sample rinse in deionized water.

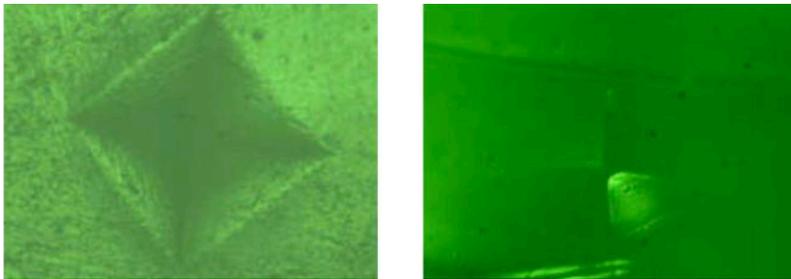


Figure 1a: Vickers imprint over the EX30 material (left) and the Smart Track sample (right)

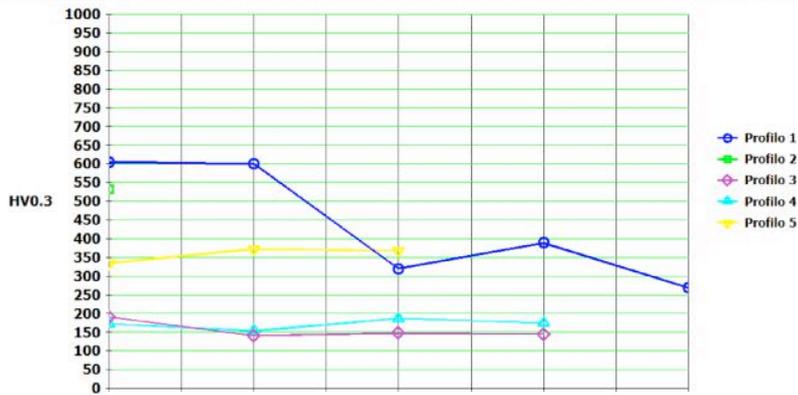


Figure 1b: A graph reporting the hardness of the different giomers. Prof 2 is a reference while the others are KE (prof1), TE (prof 3) DY (prof 4), BE (prof 5), respectively

	Smart Track	EX30
Elasticity	1.83 (N/displ_norm %)	2.48 (N/displ_norm %)
Hardness (HV)	53 ± 5	14 ± 0.7

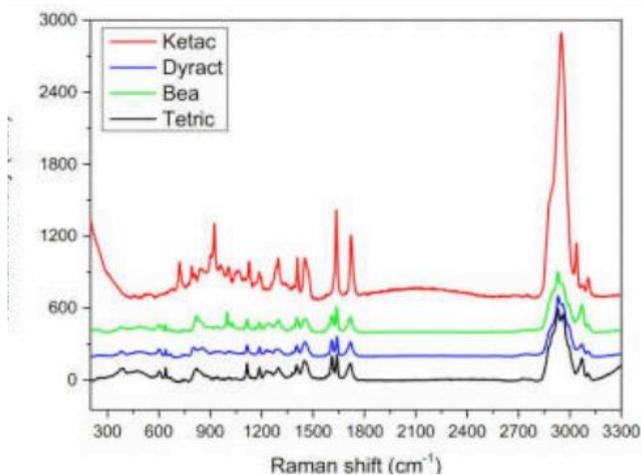


Figure 2a : The Raman spectra for the giomers before the ageing tests. The data have been stacked by intensity offsets for clarity.

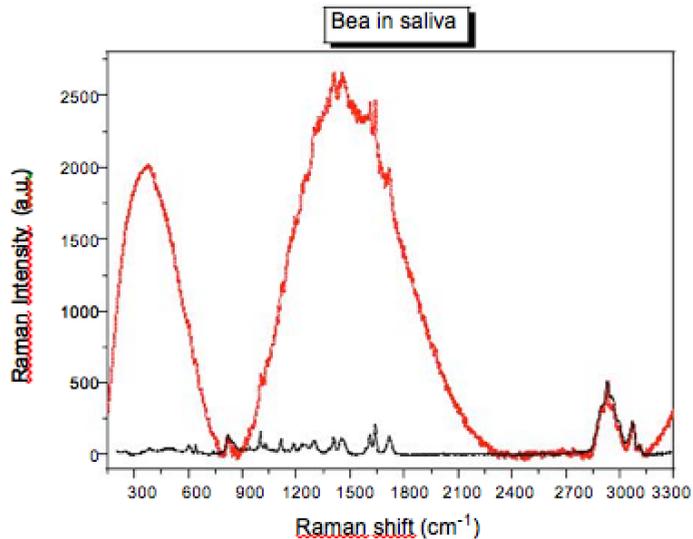


Figure 2b : The Raman spectra of a giomer (BE) after immersion in saliva for 15 days (red line). Large and intense peak related to the presence of organic residues can be detected. The spectrum collected on the clean sample surface is reported for comparison (black line).

CONCLUSIONS

The mechanical properties and the changes on the surface of new dental materials have been evaluated performing ageing tests in saliva, pbs and commercial sugary drink. A deep characterization confirms the high quality of these materials and it indicates possible improvements in their usage.

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FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY APPLICATIONS FOR THE DENTAL MATERIALS STUDY

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Keywords: FTIR, dental composite materials, bone augmentation materials

INTRODUCTION

Spectroscopy has emerged as one of the major tools for biomedical applications and has made significant progress in the field of clinical evaluation. Spectral bands in vibrational spectra are molecule specific and provide direct information about the biochemical composition. These bands are relatively narrow, easy to resolve, and sensitive to molecular structure, conformation, and environment [1]. The FTIR spectroscopy is nowadays successfully used for the characterization of dental materials, in particular to determine the conversion degree of monomers, which by polymerization are important components of dental works [2].

FTIR ANALYSIS OF DENTAL COMPOSITES

The FTIR spectrum of the uncured composite material shows the specific spectral lines of the monomers in the composite material structure. The light-curing of the materials determine the appearance on the FTIR spectra of new spectral lines corresponding to the new functional compounds formed by the organic component of the composite linking at the inorganic component. Also it finds the decreasing in intensity of the peaks corresponding to the composite monomers. Transmittance of the material also increases spectacularly as the material shows a higher degree of polymerization [3]. We used FTIR to analyze the degree of conversion of a dental

composite with the exposure time to the light source. We found the light –curing after the exposure time recommended by the manufacturer increase the degree of conversion, but only in a smaller measure that it was expected . In another study, we used the FTIR spectroscopy on samples of material subjected to staining to determine possible chemical links that may form between a coloring agent and the organic resin matrix. we used 3 colorants: coffee, food dye, red wine on 3 laboratory resin materials.

FTIR ANALYSIS OF BONE AUGMENTATION MATERIALS

FTIR spectroscopy constitutes an excellent tool to characterize the bone matrix because its main components (carbonated hydroxyapatite and collagen) absorb infrared radiation at distinct, almost complementary, regions. The process of natural biomineralization in bone is finely controlled via chemical, physical, morphological and structural mechanisms [4]. FTIR was used to provide information concerning the chemical composition and the major functional groups.

CONCLUSIONS

The main use of FTIR in dentistry is nowadays related to the degree of conversion of the dental composites showing how the seemingly simple process of resin light curing is actually a very complex, and intricate process. FTIR spectroscopy is increasingly more often used for the analysis of also other dental materials and for the synthesis and characterization of new composite materials. FTIR spectroscopy may be used also for the analysis of the oral soft and especially hard tissues and this technique may be applicable to clinical diagnostics.

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EFFECT OF HYDROXYAPATITE ADDITION ON MECHANICAL PROPERTIES OF LIGHT CURED DENTAL COMPOSITES

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Keywords: hydroxyapatite, dental composite, biocompatibility, flexural strength.

INTRODUCTION

As the main biomineral component in enamel and dentine, hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is responsible for their hardness and mechanical properties. For this reason, in the recent years hydroxyapatite has been introduced as novel bioactive reinforcing fillers in dental restorations thanks to its excellent bioactivity, biocompatibility and good mechanical performances in comparison with bio-inert inorganic particles filler [1-2]. In the last years, with the purpose to overcome limits affecting long-term durability of restorations in large stress-bearing and to obtain a balanced combination between composite morphology and mechanical performances an improvement on filler packing, optimization of filler content and development of a new innovative hybrid filler formulation become necessary [3-4]. To achieve a final consistency and specific formulation suitable for the optimal incorporation of hydroxyapatite particles mixed with silicon oxide powder fillers as well as to increase the mechanical properties, dental resin composites with different mass fractions of hydroxyapatite filler (0%, 2%, 5%, 10%, 15% and 20% wt) were prepared. The flexural characterization was performed at varying span length, in the range of 18.5 mm as stated by ISO 4049 flexural test (IFT) and 10.5 mm according to mini-flexural test (MFT). In addition, the effective enhancement of composite biocompatibility was evaluated by cell viability test.

RESULTS AND DISCUSSION

Silicon oxide reinforced bis-GMA based dental composite with different mass fractions of nano sized hydroxyapatite filler (0%, 2%, 5%, 10%, 15% and 20% wt) were prepared. The variation of flexural strength, elastic modulus and deformation against HA filler loading is shown in Table I. Each datum in the table provides the mean value of 10 measurements with standard deviation.

Table I. Failure strength, elastic modulus and deformation average values at increasing HA/glass filler ratio.

Samples	E_{IFT} (GPa)*	σ_{IFT} (MPa)*	ϵ
HA-0%	5.997(0.463)	69.02(20.52)	0.012
HA-2%	8.630(0.787)	99.83(19.63)	0.012
HA-5%	9.111 (0.211)	105.47(17.06)	0.013
HA-10%	8.788(1.448)	87.95(9.62)	0.011
HA-15%	8.687(0.568)	84.98(24.93)	0.011
HA-20%	8.697(0.882)	70.54(12.83)	0.0085

^a Mean value (standard deviation)

It is possible to observe that at low HA content, mechanical performances increase markedly with the HA weight fraction. The maximum can be observed for HA-5% evidencing a failure stress of 105.47MPa (about 52.81% higher than HA-0%) and modulus of 9.111 GPa (about 51.92% higher than HA-0%) thus demonstrating both a strengthening and toughening effect induced by the addition of HA filler.

At higher HA content the mechanical performances progressively decreased at increasing HA amount. This behaviour could be explained considering that at high HA percentage the composite is characterized by a reduced cohesive strength and higher heterogeneity which could allow a premature fracture at lower stress level.

A further increase of HA amount (HA-20%) causes premature failure of the sample at low stress level. The main reason could be researched in the presence of HA agglomerates that was observed in the resin matrix at large amount of HA, that acted as UV light scattering centres, so promoting a shielding effect on camphorquinone

activation of bulk composite resin causing a not optimal crosslinking. This process induces a significant decrease of flexural strength, about 33.12%, from the optimum value reached at HA-5%. To better evaluate the influence of such contribution in the resin elastic modulus, as values independent from span length, an extrapolation technique was used. This technique allowed to calculate the elastic modulus at different span length, by eliminating the shear deformation contribute from the apparent modulus E_r . Analogously, by the slope of interpolation line in a $1/E_r$ vs $(h/L)^2$ plot it was possible to determine the shear modulus. The flexural data are reported in figure 1. The HA-5% samples evidenced the highest apparent elastic modulus, E , (consequently the lowest $1/E$ contribute) in all range of spans. With the increase in hydroxyapatite content the apparent elastic modulus progressively decreases. An elastic modulus of about 6.729 and 9.342 GPa was calculated respectively for HA-0% and HA-2% samples. The increment rate, in comparison with the standard flexural test was about 10% for both groups. Instead a significant difference of about 30% higher than the standard one can be observed for HA-5% samples between the true elastic modulus. HA-10% and HA-15% showed an increase rate of respectively 17.23% and 23.17% in comparison with IFT values. Concerning HA-20 a modulus of 10.487 GPa can be obtained through the linear interpolation technique in comparison with the original 8697.17 MPa value (increase rate about 20%).

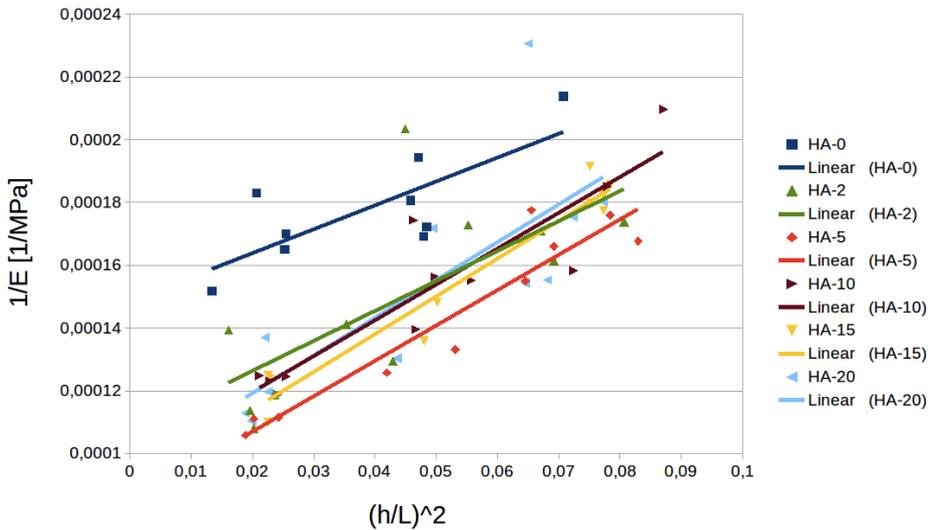


Figure 1: Trend comparison of $1/E_r$ vs $(h/L)^2$ for different hydroxyapatite mass fractions samples.

Finally the biocompatibility of the different dental composite mixture was evaluated by cell viability test. The incubation of HGF-I cells with different concentrations of various composite powders caused a dose-dependent decrease in cell viability measured by MTT assay. In particular, the resin composites with highest HA content (20%) exerted cytotoxic effects on cell cultures in comparison to control cells. This was supposed to be due to the previous mentioned shielding effect of the hydroxyapatite fillers that, at high content, reduce the degree of conversion of the matrix, consequently increasing the availability of cytotoxic uncured monomers and initiator. Among all HA composites, HA-5% demonstrated no significant cytotoxic effects.

CONCLUSIONS

This preliminary study showed how up to the 5% volume ratio of hydroxyapatite the links between micelles provide a good interaction at matrix/filler interface compared to commercial composite. On the contrary, at increasing HA content the agglomeration of the filler causes brittleness and shielding effects which cytotoxic effects. A combined IFT-MFT testing approach can be able to overcome the ISO 4049 deficiencies by providing useful information for the prediction of the mechanical behaviour of resin composites under realistic conditions of application. The MTT cell viability test showed how up to the HA-10% rate the good crosslinking of the matrix decrease the leaching of no reacted groups and thus the side effects in HGF-I cells in comparison with commercial composite formulation.

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STEM CELLS IN TISSUE REGENERATION AND OSTEOGENESIS: AN OVERVIEW

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Keywords: Osteogenesis; scaffolds; stem cells; tissue regeneration

STATEMENT OF THE PROBLEM

Surgical procedures involving the partial excision of bone, bone grafting, and fracture repair are major contributors to the healthcare burden. As a matter of example, it has been estimated that one out of three women aged >50 will experience fractures due to osteoporosis.

The repair rate of bones is dependent on the wound size: if the defect size overcomes the healing capacity of osteogenic tissues, then the fibrous connective tissue becomes dominant. Clinical approaches to bone repair include autograft and allograft transplantation; however, they have limited availability and are associated with a remarkable risk of postoperative complications.

Tissue engineering applies the knowledge of bioengineering, biology, cell transplantation, and materials science to develop biological substitutes able to restore and maintain normal function in injured bone. This tissue engineering approach often involves the use of stem cells, which are seeded into 3D scaffolds. These cells are then induced to generate new bone by the application of osteoinductive cues.

To this end, a fundamental requirement for tissue-engineered bone grafts is represented by the ability to successfully integrate within the host tissues, allowing at the same time load-bearing and tissue remodelling. Another challenge is the necessary reduced size for scaffold-tissue constructs, which limits the efficient transport of oxygen, nutrients, and metabolic wastes.

Therefore, 3D scaffolds should be designed to accommodate these requirements: their composition and physical/chemical properties must be optimized to allow the best proliferation and differentiation of stem cells. For instance, nanostructured

materials presenting peculiar properties can have a role in this process.

This presentation reviews various types of stem cell sources that have been used for tissue engineering applications, with a focus on osteogenesis.

OUTLOOK

Scaffold-based tissue engineering using stem cells is a relatively new approach. Therefore, a deep scientific knowledge of each specific stem cell type is crucial to identify how to translate them to the clinical setting. Moreover, current tissue engineering approaches are mostly working on relatively small defects, and resulting tissues are immature compared to native tissue. In addition, the long-term quality and safety of the repair should be guaranteed.

We believe that the nanotechnology-based optimization of scaffolds holds the promise to overcome at least some of these major challenges.

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WHY AND HOW BIOPHYSICAL METHODS SHOULD IMPACT ON NEXT TISSUE ENGINEERING

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Keywords: biophysical methods, bioelectromagnetic medicine, electromagnetic signalling

INTRODUCTION

Are we on the right path in actual tissue engineering? Are we missing some dimension in the understanding of tissue dynamics as for their growth, renewal, and regeneration? Are there some additional features that could improve and foster the clinical implementation of the tissue engineering techniques at the bedside? We need to enlarge the context of biology including biophysical signalling as current part of cell endogenous physiology [1] as well of cell to cell interactions in tissue and systemic adaptive behaviour [2]. An efficient and effective local and systemic adaptive behaviour is actually one of the key feature by which tissue engineering techniques can achieve their main aim in case of replacement consequent to traumas or pathologies. To these purposes, as well known, a very precise commitment is crucial to set the spatial, temporal and functional characteristics. Since biophysical signalling is, in our opinion, essential to a comprehensive and efficient tissue functioning it worth to include it as far as possible into next tissue engineering techniques [3].

RESULTS AND DISCUSSION

The occurrence of a resonance phenomenon plays the key role in mediating the effect of external electromagnetic signals on cell and tissues in bio-electromagnetic methods [4]. Remarkably the same resonance effect occurs between external electromagnetic signals and aqueous systems in both intracellular and extracellular compartments and

confirms, this way, the crucial role played by aqueous systems as one of the primary target in electromagnetic signals tissues interaction dynamics [5]. In some previous work our group has reported the effectiveness of a biophysical procedure based on a resonance effect to induce differentiation toward the cardiac phenotype of cardiospheres obtained from human cardiac biopsy [6]. As well, our group has reported evidences for the biophysical transferring to the aqueous system of the cell culture medium of the biological activity of retinoic acid on two humans cellular model, LAN5 and Teratocarcinoma, in a way that it seems to mimic its differentiation effects [7].

OUTLOOK

It is our wish that integrating the biophysical procedures into future tissue engineering we could enhance and widen their clinical applications as well as boosting their efficiency and efficacy providing, also, innovative and non-invasive integrative tools in translational medicine [8]. Moreover, it is important to take into account the electromagnetic variables in future tissue engineering with the aim of avoiding harmful interferences (electromagnetic pollution) and of implementing useful electromagnetic signals (morphogenetic fields) by means of well standardized procedures.

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THERMO-RESPONSIVE METHYCELLULOSE HYDROGELS FOR CELL SHEET ENGINEERING

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Keywords: smart materials, methylcellulose, rheology, cytocompatibility, cell sheet

INTRODUCTION

Smart hydrogels are able to change their properties in response to external stimuli, such as temperature or pH variations (i.e. thermo-responsive and pH-responsive hydrogels, respectively)¹. Methylcellulose (MC), derived from partial substitution of hydrophilic hydroxyl groups (-OH) with hydrophobic methoxy groups of cellulose, can be mixed in aqueous solutions, obtaining thermo-responsive hydrogels². These hydrogels have a reverse thermo-reversibility and undergo sol-gel transition when heated. Moreover, this sol/gel transition allows changing their affinity with solvents from hydrophilic to hydrophobic. Gelation temperature (Low Critical Solution Temperature, LCST) is affected by several parameters. In particular, MC concentration and the presence of ionic compound in the aqueous solutions are the most investigated. Ions in solution are defined as salting-out or salting-in, depending on decreasing or increasing of the LCST, respectively³. These features, together with the large availability of cellulose, the low cost of its derivatives and the biocompatibility of the material, make MC a promising biomaterial for applications in the biomedical field⁴. In particular, MC is investigated as injectable gel for soft tissues regeneration and in the innovative field of cell sheet engineering⁵. In the latter case, thermo-responsive polymers, such as

MC or poly-Nisopropylacrylamide (PNIPAAm), can be used as substrates to produce cell sheets *in vitro*. In this approach, cells adhere and proliferate on the hydrophobic hydrogel substrate at 37°C, while they spontaneously detach, preserving tight junctions and ECM, when the substrate becomes hydrophilic, switching its temperature down to 20°C⁶. Cell sheets can be applied directly on pathological tissues without cell dispersion in the surrounding tissues, as occurs in cell delivery.

This work aims to the design and physic-mechanical and biological characterization of MC-based hydrogels for applications in cell sheet engineering.

MATERIALS AND METHODS

Hydrogels were prepared preparing MC solutions with different concentration of PBS and Na₂SO₄ (or in aqueous solution, as reference, Table I). MC powder was added in the solutions preheated at 55°C to allow a homogeneous powder dispersion. The polymeric suspension was then move at 4°C for 24 h to allow a complete hydration of the MC.

[MC]	Salt [Salt]	Sample
8% w/v	//	MC-TQ
8% w/v	PBS 10g/l	MC-PBS10
8% w/v	PBS 20g/l	MC-PBS20
8% w/v	Na ₂ SO ₄ 0.05M	MC-Na005
8% w/v	Na ₂ SO ₄ 0.1M	MC-Na01

Table I: Composition of the MC-based hydrogels investigated in this work

The mechanical characterization of MC based hydrogels was performed using a rotational rheometer (AR-1500 TA Instruments). The LVR (Linear Viscoelastic Region) was determined with strain sweep tests, and then temperature sweep tests were performed to determine the sol-gel transition temperature of all the considered hydrogels. The mechanical parameters evaluated, varying the temperature, were: conservative modulus (G'), viscous modulus (G'') and complex viscosity (η^*).

Stability and hydrophilicity of MC-based hydrogels were investigated by swelling tests in distilled water at 37°C. Hydrogels ($n = 3$ for each formulation) were weighted in dry condition at the beginning of the test, and at different time points up to 7 days in wet conditions. At each time point, swelling was calculated as difference between wet

and dry sample weight, normalized for the dry weight.

In vitro cytotoxicity tests were performed with murine fibroblasts L929 cell line; while, for the cell sheets detachment test, NIH-3T3 mouse embryo fibroblasts modified with a gene for the expression of Green Fluorescent Protein (GFP) were selected. The *in vitro* indirect cytotoxicity of hydrogels was investigated to exclude the possible toxicity of the solution salts and their possible release into the culture medium. Samples (Table I) were placed in wells of a culture multiwell plate (48 wells), adding complete DMEM, and left in contact for 24 and 48h. L929 cells (1×10^5 cells/ml) were cultured using eluates as culture medium. After 24 h, cells viability was investigated by Alamar Blue colorimetric assay.

NIH-3T3 cells (1.45×10^6 cells/ml) were seeded on all the investigated hydrogels and cultured for 48 h. Cells viability was evaluated with XTT colorimetric assay. Once obtained, cell sheets were detached from the substrate, moving the multiwell at 4°C. Then, cell sheets were taken with a pipette and moved either on electrostatic glass slides for fluorescence microscopy observations (phalloidin was used to stain actin filaments) or into a new multiwell (12 wells) to evaluate adhesion properties and cells proliferation on the new substrate with optical microscopy. Furthermore, NIH-3T3 fibroblast cells were seeded on an appropriate PTFE mold (Figure 1), to obtain ring cellular sheets.



Figure 1: PTFE mold used to obtain ring cellular sheets

RESULTS AND DISCUSSION

All analysed samples showed no macroscopic differences at 20°C (Figure 2). MC is, in fact, soluble in water due to the hydrogen bonds formation between water molecules and hydrophilic groups of MC chains (-OH), and it leads to the formation of a transparent hydrogel. Instead, at 37°C, samples MC-Na0I (Figure 2b), MC-PBS20

and MC-Na005 exhibited a transition so that they appear opaque and hydrophobic. There is no macroscopic change for MC-TQ sample (Figure 2d) in comparison to the same hydrogel observed at 20°C.

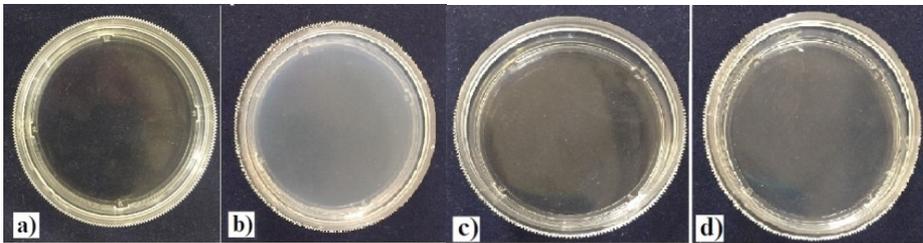


Figure 2: Macroscopic observations of MC-Na01 (a, b) MC-TQ (c, d) at 20°C and at 37°C.

From initial tests for the determination of MC-based hydrogels LVR, it has been decided to use a strain of 0,5% in the temperature sweep tests. At 20°C, G' and G'' showed a high stability until strains over 50%; instead, at 37°C, no proportionality between stress and strain was detected for strains around 1%. Temperature sweep test showed G' and η^* behaviour versus temperature; both rheological parameters reduced at the beginning of the test and then, they drastically increase (Figure 3) when the gelation process starts. This increase started at lower temperature when a 2°C/min heating rate was used in comparison the 5°C/min heating rate. G' and η^* values were higher for 2°C/min curves, than 5°C/min ones. Moreover, samples heated at 2°C/min at the end of the test reached a plateau, that is not detectable for samples heated at 5°C/min since the gelation process has not ended yet using this testing parameter (Figure 3, complex viscosity trends for sample MC-PBS20 is reported). From the obtained curves, sol-gel transition temperatures of MC-based hydrogels were calculated (Table 2). Transition shifts to lower temperatures in comparison with MC-TQ sample if salting-out ions are added in solution; higher the saline solution concentration, higher is this reduction. In particular, differences between MC-TQ and samples with the highest salt concentrations (MC-Na01 and MC-PBS20) are significant ($p < 0.05$).

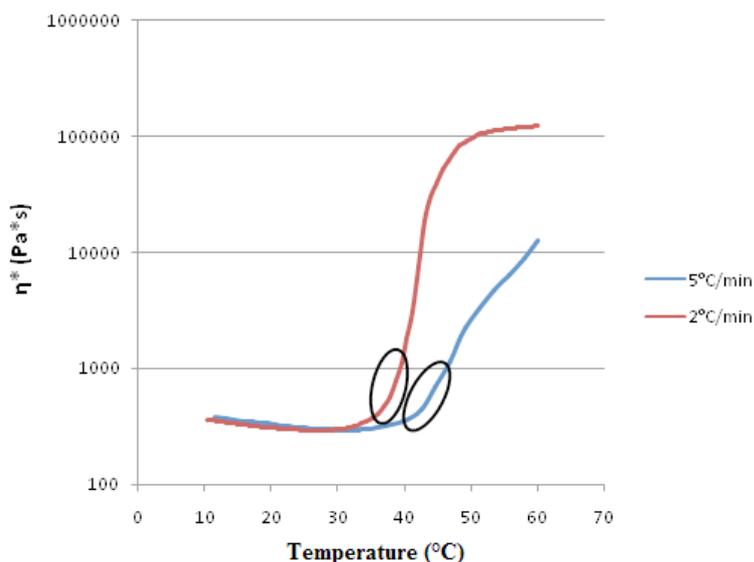


Figure 3: Complex viscosity vs temperature for MC-PBS20 sample

	5°C/min (°C)	2°C/min (°C)
MC-TQ	43.9 ± 0.5	38.7 ± 0,5
MC-PBS10	43,0 ± 0.7	39.9 ± 0.8
MC-Na005	43,5 ± 0.4	37,9 ± 0.3
MC-PBS20	41.3 ± 0.7	36.9 ± 0,3
MC-Na01	39.3 ± 0.1	37.2 ± 0.5

Table 2: Gelation temperatures obtained from G' vs temperature curves

All tested hydrogels were stable until 4 days in distilled water; in particular, MC-Na01 was stable until 7 days. Moreover, swelling in culture medium of the hydrogels ranged from 150% for the most hydrophobic samples (i.e., MCNa01 and MC-PBS20) to over 200% for the most hydrophilic ones (i.e., MC-TQ, MC-PBS10 and MC-Na005).

The cytotoxicity test demonstrated, by Alamar Blue assay, that L929 cells that have been in contact with MC-based hydrogels eluates exhibited viability values comparable with those of positive control (viability for eluates extracted after 48h is represented in Figure 4). Finally, the MC hydrogels here investigated did not release any toxic substances for cells.

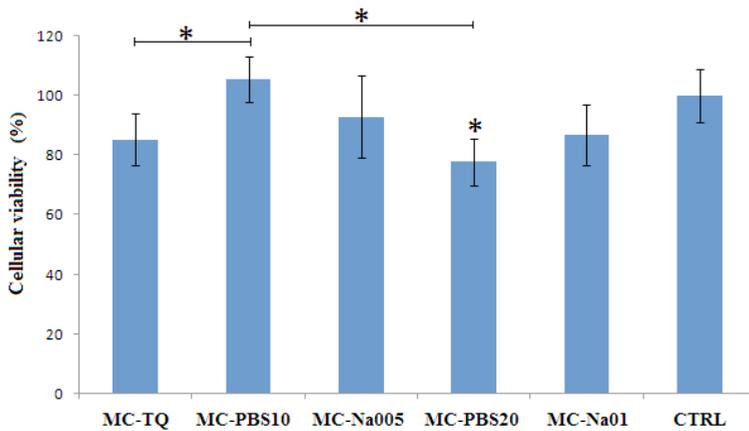


Figure 4: Viability of L929 cells cultured with eluates (culture medium in contact with hydrogels for 48h). (*) significant difference ($p < 0,05$)

NHI-3T3 were seeded onto the MC hydrogels (Table I) for 48 h, then the multiwells were moved at 4°C, and after 5 min, cell sheets started to gradually detach till the complete detachment after 20 min. No macroscopic differences were observed among the sheets detached from different hydrogels (Figure 5). Differences were found for detachment efficiency, expressed as the percentage of intact sheets on the total of detached ones; MC-Na005 and MCPBS20 hydrogels show a 100% efficiency, thus resulting very promising to be produced in large scale, where time and cost production optimization is required.

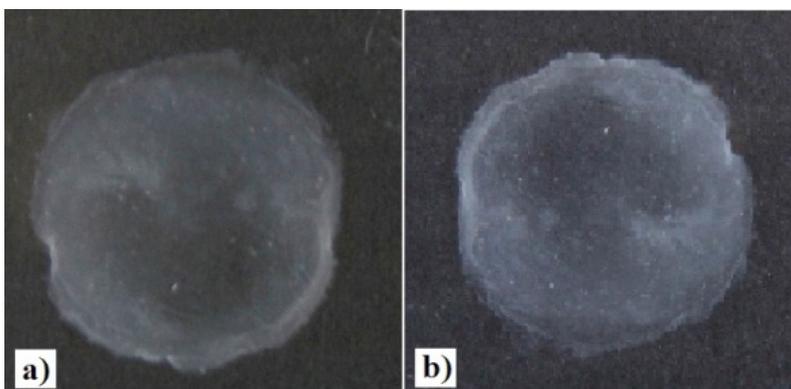


Figure 5: Macroscopic comparison between cell sheets detached from (a) MC-Na005 and (b) MCPBS20 hydrogels

At fluorescence microscope, a high cellular density (stained with green) can be noticed for sheets obtained onto MC-PBS10, MC-Na005 and MCNa01 (Figure 6a) compared to the ones detached from MC-TQ and MC-PBS20 (Figure 6b) that showed a lower cell density. To validate this hypothesis, it would be necessary to repeat this experiment, increasing samples number. MC-Na005 and MC-PBS20 resulted as the optimal MC hydrogels compositions; in fact, some of the sheets detached from the other types do not adhere to the new substrate, showing an insufficient ECM production.

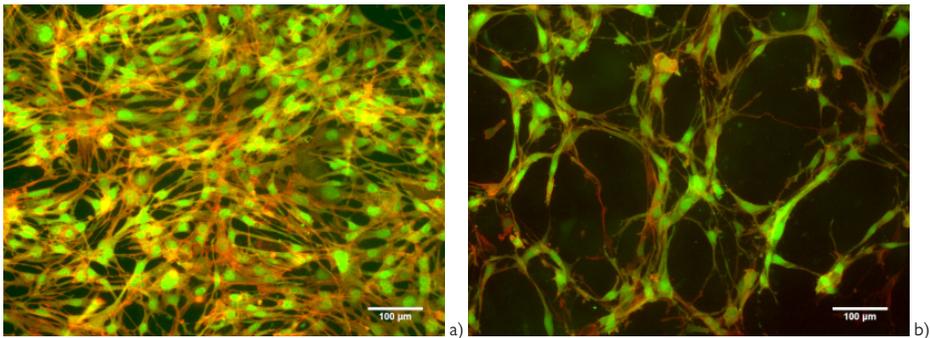


Figure 6: Fluorescence microscopy images of cell sheets detached from (a) MC-Na01 and (b) MC-PBS20. Scale bar = 100µm.

After 6 days of culture in a new culture well, cells of the cell sheet started to move from the sheet edges and to proliferate, colonizing the well bottom where the cell sheet was moved after the detachment, showing a good viability. This result is encouraging for future sheets application in regenerative medicine; the produced ECM would, in fact, ensure the cell sheet adhesion to the damaged tissue, allowing cells to proliferate and regenerate the tissue.

O-ring cell sheet were obtained considering the MC hydrogel formulations that gave better results in the previous test (i.e. MC-Na005 and MC-PBS20). Staining of the cell sheet demonstrated the feasibility of the production of ring cell sheets onto both the formulation (Figure 7a-b). Even after moving of the ring cell sheet into a new culture well, cell maintained their junctions and the round shape (Figure 7c).

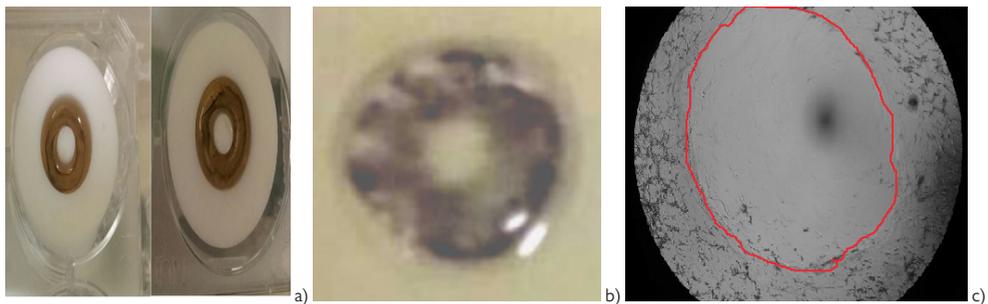


Figure 7: Macro images of o-ring cell sheets detached from (a) MC-Na01 and (b) MC-PBS20; (c) o-ring cell sheet moved into a new culture well.

CONCLUSIONS

MC-Na005 and MC-PBS20 hydrogels resulted as the optimal formulations; in fact, both these ones have a LCST slightly below 37°C, ideal for cell sheet engineering applications. Moreover, they showed a 100% cell sheet detachment efficiency, and those sheets showed good adhesion to new substrates, compactness and cohesion. Among these two optimal hydrogels, MC-Na005 could be used and developed in cell sheet engineering, since the higher cellular density emerged from fluorescence microscopy qualitative analysis. The possibility of obtaining different shape of the cell sheet represents a huge advantage for the regeneration of different tissue, for example for small caliber vascular vessels.

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POSTER

INCORPORATION OF MESOPOROUS GLASS PARTICLES IN A RESORBABLE GLASS FIBROUS SCAFFOLDS: A STRATEGY TO IMPROVE ITS BIOACTIVITY

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Keywords: phosphate glass; glass fibres; bone scaffold; mesoporous bioactive glass; mesoporosity

INTRODUCTION

Porous 3-D scaffolds are proposed as an alternative to the use of bone grafting technique in the situations of not-sufficient spontaneous bone regeneration.

In this work, resorbable phosphate fibres and bioactive mesoporous particles (*i.e.* specific surface area up to 800 m²/g, adjustable pore size between 2 and 50 nm, large pore volume [1]) with different morphology and size were selected to combine the resorption property of the fibres with bioactive property of the powders.

TiO₂-containing phosphate glass (TiPS_{2.5}) fibres (diameter of 110 μm) were fabricated following the preform drawing technique, using a drawing tower, as described elsewhere [2]. A dense silica-based bioactive glass (CEL2) [3] was produced by melt quenching as reference sample. Spherical micro-sized mesoporous glass particles based on SiO₂-CaO system (SD_MBG) were produced combining sol-gel method with the aerosol-assisted spray-drying technique [4]. Cu-containing (85SiO₂-13CaO-2CuO, % mol, referred as Cu_BGn2%) mesoporous glass nanoparticles, with antibacterial properties, were synthesized by an ultra-sound assisted sol-gel method. To produce the fibrous scaffolds,

the selected powder and phosphate glass fibres cut at precise length were placed in a beaker containing 2 ml of ethanol. After ethanol evaporation, the powder/fibre mixture was then randomly placed inside a zirconia cylindrical mould. After the thermal treatment, scaffolds were analyzed with micro-CT in order to investigate their inner structure. By soaking them in SBF, their ability to form hydroxyapatite was investigated. Scaffolds morphology before and after immersion in SBF was studied by FESEM.

RESULTS AND DISCUSSION

FESEM micrographs show that CEL2 ($<20 \mu\text{m}$) are not well incorporated on fibre surface. On the contrary, SD-MBG (Figure 1.a-b-d) and Cu_BGn2% particles homogeneously cover the whole surface. Micro-CT analysis did not reveal the presence of powder agglomerates for all the observed scaffolds and showed a homogeneous porosity of 58 vol.% for CEL2/fibre scaffold, of 53 vol.% for SD_MBG/scaffold (Figure 1.c) and 33% for Cu_BGn2%/scaffold.

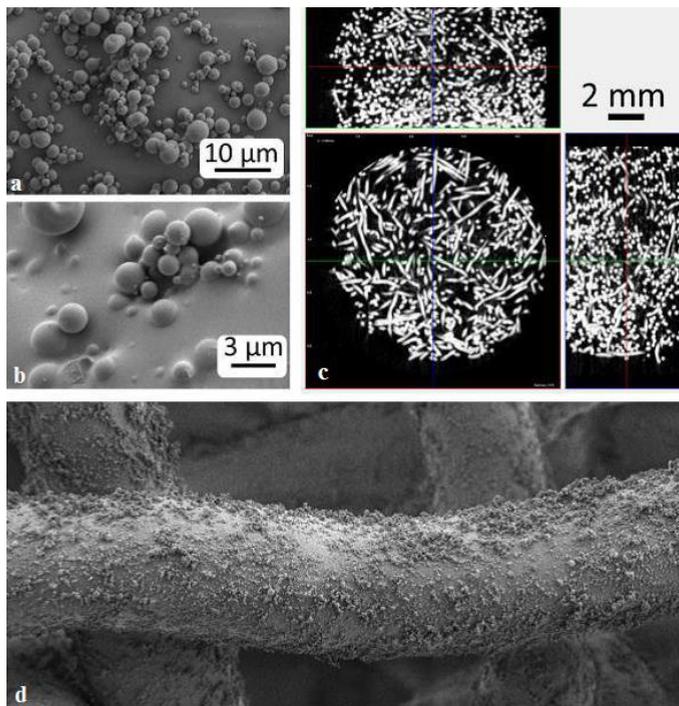


Figure 1: a-b-d) FESEM micrographs results of SD_MBG/fibre scaffold, c) micro-CT result of SD_MBG/fibre scaffold [5].

In CEL2/fibre scaffolds glass particles mostly detached during soaking in SBF leaving some pits on the fibre surface: FESEM analysis (Figure 2.a) revealed few particles still anchored to the scaffold surface after 7 days, indicating that CEL2 particles were not effectively anchored to the fibre surface. At variance, SD-MBG (Figure 2.b) and Cu_BGn2% particles showed a better inclusion and after 7 days in SBF they were clearly visible on the surface of the scaffolds and after 1 day of soaking in SBF, they appeared (Figure 3) fully covered with HA layer, showing the typical “cauliflower-like” morphology.

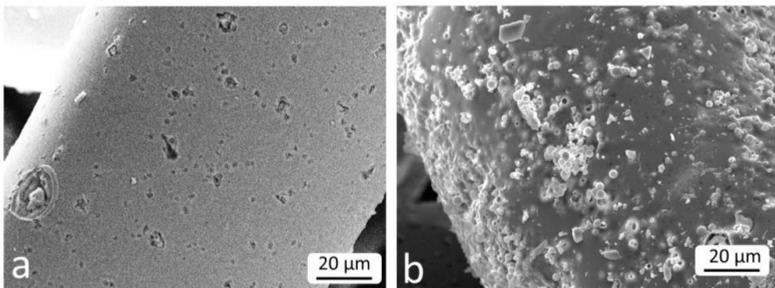


Figure 2: FESEM micrographs of CEL2/glass fibre scaffold (a) and SD-MBG/glass fibre scaffold after 7 days in SBF [5].

This difference can be due to the size and shape of the SD-MBG particles, which are smaller and spherical-shaped, thus allowing a more efficient covering of the fibre surface and an easier incorporation upon sintering.

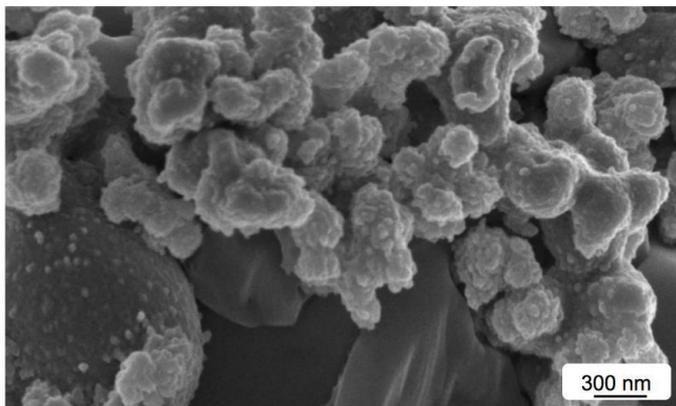


Figure 3: FESEM micrograph of Cu_BGn2%/scaffold after 1 day in SBF.

CONCLUSIONS

The incorporation of mesoporous bioactive glass powder in the phosphate glass fibrous scaffold resulted to be a very interesting strategy to impart multifunctional properties to the scaffold. Their fast bioactive response is due to their mesoporosity: it involves a high surface area available for ion exchange which is responsible for the glass bioactivity. These promising results encourage further investigation in order to fully exploit the ability of mesoporous particles to act as a system for smart release of therapeutic ions and drugs.

ACKNOWLEDGEMENT

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ELECTROPHORETIC DEPOSITION OF SR-CONTAINING MESOPOROUS BIOACTIVE GLASS PARTICLES PRODUCED BY SPRAY-DRYING

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Keywords: Mesoporous bioactive glass, spray-drying, strontium, scaffold, tissue regeneration.

INTRODUCTION

Mesoporous bioactive glasses (MBGs) are gaining increasing interest in the biomedical field thanks to their exceptional textural characteristics (high surface area, high pore volume and highly ordered mesoporosity). These properties lead to an improved apatite kinetics formation, which allow these glasses to be successfully applied in bone tissue regeneration [1].

In this work we adopted an aerosol-based spray drying process in order to have high control and reproducibility over the morphology of particles. In order to increase their regenerative potential, the particles have been doped with strontium, element known for its osteogenic and bone antiresorptive properties [2].

Later the particles have been deposited by electrophoretic deposition (EPD) on glass-ceramic scaffolds fabricated by the polymer sponge replication method. EPD is a versatile technique which allows an easy control of the thickness of the deposited film through simple adjustment of the applied voltage and the deposition time. The scaffolds, based on a quaternary silicate glass (SCNA, $\text{SiO}_2\text{-CaO-Na}_2\text{O-Al}_2\text{O}_3$ oxide system), have good mechanical properties but low bioactivity [3]. Thanks to MBG particle deposition, they acquire a pronounced bioactive behaviour, thus becoming an excellent solution for bone tissue regeneration.

RESULTS AND DISCUSSION

MBGs synthesized with the aerosol-based spray-drying process have a basic composition on the SiO_2 -CaO system and have been doped with the 1% molar of strontium (SD_Sr1). FESEM image of particles (Figure 1 A) shows micro-sized spherical particles, with size mostly ranging between 500 nm and 5 μm .

N_2 adsorption analysis gives back a high specific surface area value, 160 m^2/g , and a pore size distribution between 5 and 9 nm, which confirms the mesoporosity of the sample. Strontium incorporation inside the binary composition does not modify the bioactive behaviour of the glass: after 14 days in SBF nanoparticles are completely covered by a layer of hydroxyapatite (Figure 1 B).

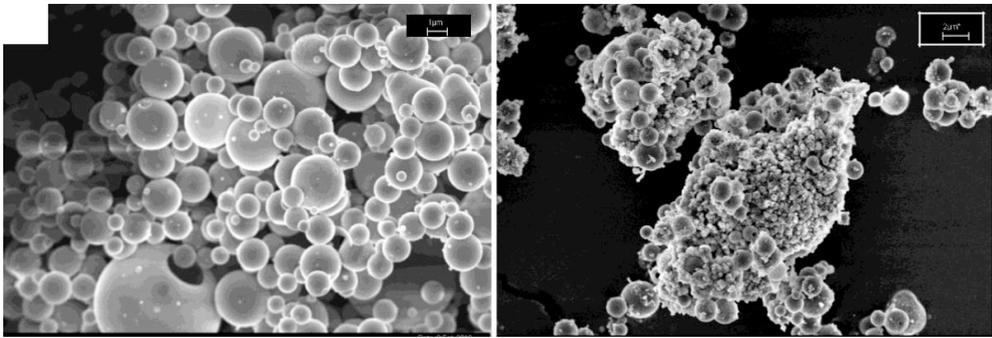


Figure 1: FESEM micrographs of Sr-doped MBGs prepared by aerosol-assisted method as synthesized (A), after immersion for 14 days in SBF (B)

The EDS quantitative analysis shows that the amount of strontium effectively incorporated in the microparticles was 70% of the theoretical one, probably because of the high dimension of the ion which hinders its entrance into the glass network. Nevertheless, most of the Sr incorporated has been released after 14 days of immersion in SBF, as the coupled plasma-atomic emission spectrometry (ICP-AES) reveals. On the basis of literature data, the released concentrations are suitable for inducing osteogenesis [4].

EPD has been performed in ethanol, applying a voltage of 120 V for 5 minutes. The scaffolds, being not conductive, have been suspended between two stainless steel electrodes through a clamp. A dispersant (TEA, triethanolamine) has been used to keep the particles in suspension during the whole deposition time. The deposited layer was abundant but not uniform on the scaffold surface (Figure 2 A). After immersion

for 7 days in SBF, hydroxyapatite formation has been observed on the surface of the microparticles deposited on the scaffold struts (Figure 2 B). This demonstrates that MBGs not only maintain their bioactivity after deposition but also transfer this property to scaffolds.

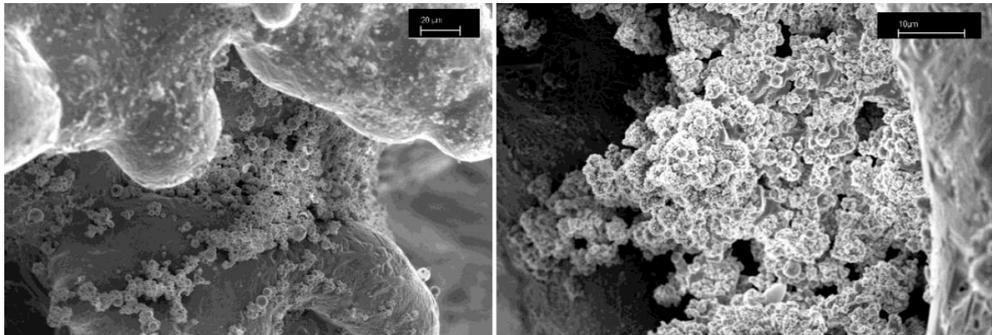


Figure 1: SCNA scaffold after MGB deposition by EPD (A) and after immersion for 14 days in SBF (B)

CONCLUSIONS

MBGs synthesized with aerosol-based spray-drying process and doped with strontium have excellent textural properties and a bioactive behaviour. After electrophoretic deposition, they maintain these properties and consequently they improve the bioactivity of SCNA scaffolds, which initially are almost biologically inert.

In this way we demonstrate that it is possible to obtain a successful construct for bone tissue engineering with both excellent regenerative and mechanical properties.

ACKNOWLEDGEMENT

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SYNTHESIS AND CHARACTERIZATION OF CERIUM-CONTAINING MESOPOROUS BIOACTIVE GLASS NANOPARTICLES FOR BIOMEDICAL APPLICATIONS

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Keywords: bioactive glass nanoparticles, mesoporous, cerium, surface modification

INTRODUCTION

Mesoporous bioactive glass nanoparticles (MBGN), owing to their small size, high specific surface area (SSA) and uniform shape, are attracting increasingly attention in a large number of biomedical applications [1,2]. In comparison to conventional solid bioactive glasses, MBGN are able to carry functional biomolecules (e.g. antibiotics and growth factors) more effectively for intended applications. Recently, the incorporation of therapeutic ions into materials is becoming a fascinating strategy to endow the materials additional functionalities [3], as the therapeutic ions are able to be angiogenic, antibacterial and anticancer, depending on the type and amount of the ions [3,4]. BG are promising vehicles to deliver the therapeutic ions, as their composition and degradation rate can be conveniently tailored in comparison to crystalline biomedical materials (e.g. apatite).

Cerium oxide nanoparticles (CeN) have pro-oxidant and anti-oxidant effects on different cell systems or organisms [5]. They can initiate angiogenesis by modulating the intracellular oxygen environment to stabilize hypoxia and consequent increase the production of vascular endothelial growth factor (VEGF) [6]. The CeN can also

accelerate the healing of full-thickness dermal wounds by enhancing the proliferation and migration of keratinocytes, fibroblasts, and vascular endothelial cells [7]. Moreover, the increased $\text{Ce}^{3+}/\text{Ce}^{4+}$ ratio on cerium oxide nanoparticles can induce higher catalytic activity towards regulating intracellular oxygen, which in turn leads to a more effective wound healing [5].

On the basis of the above rationale, cerium-doped MBGN (Ce-MBGN) are considered to be a promising material for bone regeneration and wound healing. Additionally, Ce-MBGN can also deliver therapeutic biomolecules to assist the achievement of bone regeneration or wound healing. In this study, the Ce-MBGN were synthesized via a two-step process, in which as-synthesized MBGN were doped by Ce ions using a surface modification routine. In this routine, the as-synthesized MBGN were soaked in cerium nitrate ethanol solutions under different conditions (e.g. different concentration of soaking solution or temperature). Optimized processes for the introduction of Ce ions were determined by evaluating the morphology and composition of the resultant particles. The morphology and structural characteristics of Ce-MBGN were characterized, and their apatite-forming ability was evaluated by soaking the materials in simulated body fluid (SBF). Finally, the ion release behavior, textural characteristics, and the cytotoxicity of Ce-MBGN are being investigated. More detailed results will be presented in the conference.

RESULTS AND DISCUSSION

Mesoporous bioactive glass nanoparticles with a nominal composition of 70S (70SiO₂-30CaO by mol%) were successfully synthesized via an emulsion/sol-gel based method as described in a previous study with slight modification [8]. As can be seen in Figure 1a, the synthesized MBGN showed a sphere-like shape and a mean particle size of 200 nm with mesopores (5-20 nm) on their surface. These particles were highly dispersed, which is beneficial for their further biomedical applications as building blocks for nanocomposite fabrication.

Since cerium nitrate may be converted into CeN under basic conditions [6], it is therefore a challenge to synthesize Ce-MBGN when basic substances are available as catalysts. Unfortunately, silica-based nanoparticles are usually produced under basic conditions. The nanoparticles can repel each other in a basic environment due to their highly negatively charged surface, so as to maintain the dispersion of particles. In order to introduce cerium to MBGN while at the same time maintaining

the dispersion of the particles, we used a post-modification routine to incorporate Ce ions into the MBGN, as described in our previous study [9]. In order to evaluate the effects of modification conditions on the morphology and composition of the MBGN, we soaked the MBGN in cerium nitrate ethanol solutions (the concentration of 0.2M or 0.5M) at 25 or 80°C for 1 day or 3 days. The results indicate that different modification conditions could lead to Ce-MBGN with various morphological and compositional characteristics. As shown in Figure 1, smaller particles clustering MBGN are observed on the samples modified at 80°C (Figure 1b), suggesting that the high temperature may promote the formation of CeN. In a comparison, no obvious CeN are seen in the MBGN treated at 25°C (at either 0.2M or 0.5M for 1 day or 3 days) (Figure 1c-d). Furthermore, the modified MBGN still maintained their original shape and dispersion (see Figure 1).

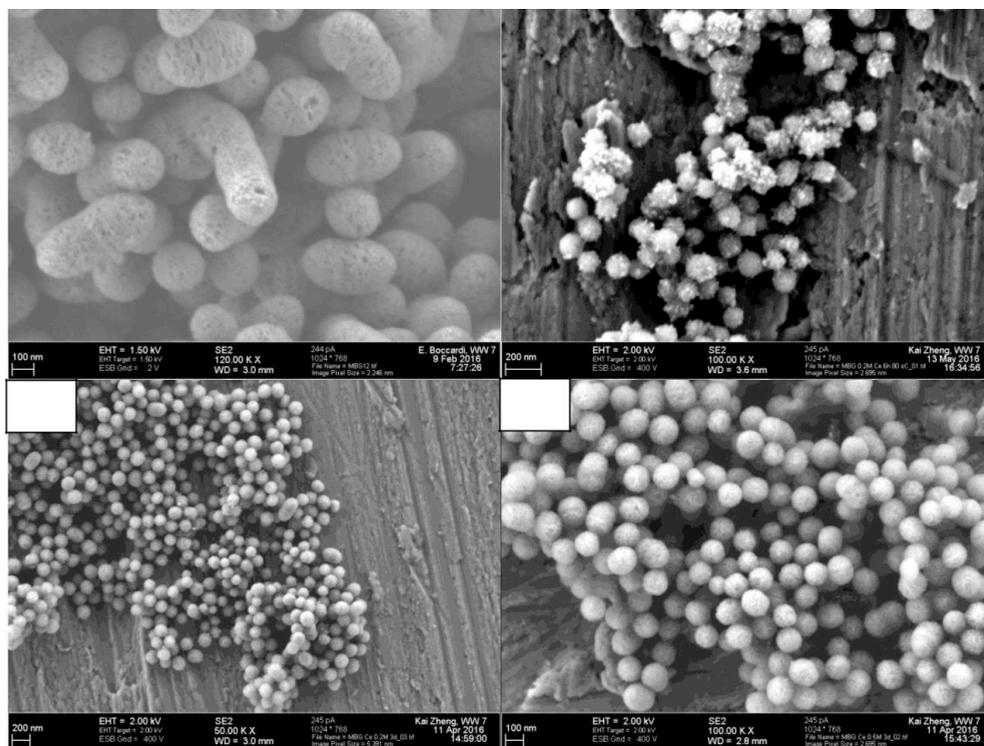


Figure 1: Representative SEM images of the synthesized nanoparticles before and after the modification. (a) The as-synthesized MBGN; (b) the MBGN soaked in 0.2M cerium nitrate ethanol solution for 24 h at 80°C; (c) the MBGN soaked in 0.2M cerium nitrate ethanol solution for 24 h at 25°C; (d) the MBGN soaked in 0.5M cerium nitrate ethanol solution for 24 h at 25°C.

The EDS results confirm the presence of cerium in the particles after the modification, indicating these particles have been converted to Ce-MBGN. Notably, the incorporated amount of Ce could be controlled by tailoring either the concentration of cerium nitrate ethanol or the soaking time. The FTIR results of the Ce-MBGN demonstrate the typical spectra of silicate materials, but no significant difference could be found between the particles before and after the modification. Importantly, the XRD results confirm the amorphous nature of the Ce-MBGN, which indicates that no crystalline CeN formed in the course of the modification process. The MBGN are highly bioactive, as apatite could form on MBGN pellets after immersion in SBF for 8 h. The Ce-MBGN still possess good apatite-forming ability; the apatite formed on Ce-MBGN pellets after immersion in SBF for 1 day, which was confirmed by SEM, EDS, XRD and FTIR.

The ion release profile, the influence of the modification on the SSA of the particles, and the cytotoxicity of Ce-MBGN are being investigated, as these characteristics are significant to the effective applications of Ce-MBGN. More detailed results will be presented in the conference.

CONCLUSIONS AND OUTLOOK

Cerium can be incorporated into MBGN via a convenient post-modification routine, which may avoid the formation of CeN by carefully controlling the modification conditions. By using this strategy, the dispersion of the MBGN (SiO_2 -CaO composition system) is able to be maintained, while the morphology of particles is not significantly affected. In addition, the incorporated amount of Ce could be controlled by tailoring either the concentration of cerium nitrate ethanol or the soaking time. The EDS results confirmed the presence of cerium in Ce-MBGN, while the XRD assay indicated that no detectable crystalline CeN were present in the Ce-MBGN. The Ce-MBGN exhibited a rapid apatite formation, which suggests their bone-bonding capability. The ion release profile and the cytotoxicity of the Ce-MBGN, as well as the influence of the modification on the SSA of the particles are being investigated. Furthermore, the valence states of Ce in Ce-MBGN will be determined, as the valences states of Ce is significant to the anti-oxidant and cell biological properties of Ce-containing materials. In summary, this modification is a convenient routine to incorporate Ce into MBGN; the Ce-MBGN are promising materials for bone regeneration or wound healing.

ACKNOWLEDGEMENT

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MORPHO-STRUCTURAL CHARACTERIZATION AND ANTIBACTERIAL RESPONSE OF COPPER CONTAINING SOL-GEL SILICATES

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Keywords: sol-gel, Cu, antibacterial, UV-vis, TEM

INTRODUCTION

Bioactive glasses represent a class of bioactive materials that has a very high potential for hard and soft tissue regeneration applications⁽¹⁾. It has been found that by adding specific metal ions one can create a multifunctional material conferring to the well-known qualities of silicate glasses⁽²⁾ a few other important characteristics such as antibacterial and angiogenic properties. Copper ions have been reported to be essential components of the angiogenic response and very active antibacterial agents with low toxicity and good stability⁽³⁾.

The aim of this study was to evaluate the antibacterial response of the $\text{SiO}_2 \cdot \text{CaO} \cdot \text{P}_2\text{O}_5$ bioactive material with CuO content between 0.5 and 4 mol%. The compositions were synthesized through sol-gel method. The obtained materials were further investigated by X-ray diffraction (XRD) and Fourier Transform Infrared spectroscopy

(FT-IR). Morphological characterization of the formed CuO particles within the materials matrix has been done by means of UV-Vis absorption spectroscopy and transmission electron microscopy (TEM), while the pores assessment was performed by sorption measurements. In order to obtain the release of Cu ions, inductively coupled plasma mass spectrometry measurements (ICP-Q-MS) was performed. The antibacterial activity of samples with CuO content have been made on *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains.

RESULTS AND DISCUSSION

The X-ray diffraction showed a predominant amorphous character and FT-IR spectroscopy evidenced the presence of absorption bands characteristic for bioactive silicate based glasses. UV-Vis absorption measurements were additionally performed. The samples containing CuO present a dominant broad band at around 800 nm characteristic to d-d transitions of Cu²⁺ in octahedral coordination⁽⁴⁾. As anticipated, this band increases in intensity as the copper content becomes higher. The release in percentage of Cu in 24 h, obtained by ICP-Q-MS was pointed to be initial concentration dependent, the higher the initial content the higher the amount released in 24 hours. TEM analyses detect a particularly amorphous porous structure with presence of metallic Cu content.

The Minimum Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC) were performed as standard procedure to identify the antibacterial activity of our materials. Regarding the inhibitory effect, that was more significant on *Pseudomonas aeruginosa* strain in all concentrations studied, withal *Staphylococcus aureus* revealed a much more resistance in 1.5, 2.5 and 4 mol% CuO concentrations, however, it was effectively combated more even than gentamycin did (Figure 1a). MBC was also determined, all sample concentrations proving again a very good activity on both *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains showing a visible high activity in sample with x=0.5 mol% and a partial efficiency when the CuO content of glass-ceramic samples was between 1.5 and 4 mol% (Figure 1b).

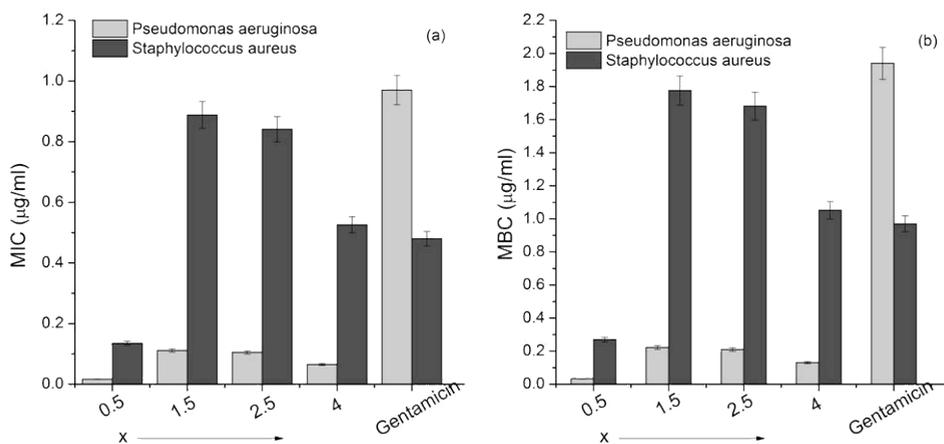


Figure 1: Minimum inhibitory concentration (MIC) (a) and minimum bactericidal concentration (MBC) (b)

CONCLUSIONS

In terms of morpho-structural characteristics the studied samples proved very good qualities for further tissue engineering applications. The Uv-vis and TEM analysis showed that all samples contain metallic Cu. The ICP-Q-MS measurements indicate a positive correlation between ionic release and antibacterial activity. This connection has demonstrated that the antibacterial efficiency of the samples increases with increasing of CuO content, *Pseudomonas aeruginosa* being killed more efficiently than *Staphylococcus aureus* strain.

ACKNOWLEDGEMENTS

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BIOCOMPOSITES AND BIOCERAMICS BASED ON RESORBABLE CALCIUM PHOSPHATES WITH $Ca/P \leq 1.5$

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Keywords: biocomposites, 3D printing, calcium phosphates, resorbability, osteoconductivity

INTRODUCTION

Modern regenerative medicine has a great need in resorbable bioactive composite materials for bone implants. Biodegradable polymers (polycaprolactone (PCL), polylactide (PLA)) filled with resorbable calcium phosphate (with the ratio $Ca/P \leq 1.5$, e.g., amorphous mixed-anionic and tricalcium phosphates) can serve as such implants. Imperative feature of the materials is specific macroporous architecture (osteoconductivity) created by 3D-printing. In the case of thermal extrusion technique of 3D-printing (or, fused deposition modeling – FDM™), it is necessary to fabricate composite cords polymer/calcium phosphate filler with uniform distribution of phosphate particles inside thermoplastic prior to printing and to elaborate printing regimes [1]. Another thrust of problems is structured around hydrophobicity of the vast majority of the polymers and, therefore, the need in modification of their surface. The aim of this work is to create bioactive macroporous composites based on calcium phosphate and biopolymers, as well as ceramic implants of predetermined complex shape, based on the mixed anionic calcium phosphates. The tasks of the work included

(i) synthesis and physico-chemical studies of amorphous $(Ca_9(PO_4)_6 \cdot xH_2O)$, ACP and amorphous mixed anionic $(Ca_{3-x}(P_2O_7)_x(P_6O_{18})_{1-x})$, $x = 0.2, 0.4, 0.6, 0.8$, maACP) calcium phosphates, (ii) fabrication multiphase dense bioceramics based on maACP, (iii) 3D-printing of macroporous biocomposites (β -TCP/poly(ϵ -caprolactone), β -TCP/poly(D,L-lactide) for bone implantation, and (iv) modification of the composite surface.

RESULTS AND DISCUSSION

ACP and maACP were obtained by precipitation from solutions by the following reactions at 10°C, pH > 10:

1. $9Ca(NO_3)_2 + 6(NH_4)_2HPO_4 + 6NH_4NO_3 \rightarrow Ca_9(PO_4)_6 \cdot yH_2O \downarrow + 18NH_4NO_3 + 3H_2O$
2. $9CaCl_2 + 6Na_2HPO_4 + 6NaOH \rightarrow Ca_9(PO_4)_6 \cdot yH_2O \downarrow + 18NaCl + 3H_2O$
3. $(3-x)CaCl_2 + (1-x)Na_6P_6O_{18} + xNa_4P_2O_7 \rightarrow Ca_{1-x}(P_2O_7)_x(P_6O_{18})_{1-x} \downarrow + 2(3-x)NaCl$

It was found that ACP particle agglomerates obtained by precipitation from the “chloride” solution method, have a smaller average size (6.4 times) compared to the “nitrate” synthesis method. By thermal treatment (with T = 500°C, 700°C and 900°C in during 10 hours) of pressed pellets maACP multiphase dense bioceramics were obtained. Composite cords were molded with a different ratio of β -TCP to biopolymers. Using Fused Deposition Modeling (FDM) technology of 3D-printing, 3D periodic structures with mesh size 16.5×16.5×4.5 mm; 30×30×3.5 mm, 10×10×2 mm, and more complex shape implants were fabricated. It was demonstrated hydrophilicity changing of the composite surface modified by plasma treatment (500V, 5mA, AC, 5-15 minutes) and soaking in 5•SBF-solution as well as by combination of methods above.

CONCLUSIONS

Thus, we obtained macroporous bioactive composites based on calcium phosphate and biopolymers as well as ceramic implants based on mixed-anionic calcium phosphates with predetermined complex architecture. It was shown that osteoconductive composite implants made of degradable polymer and bioresorbable calcium phosphate can be fabricated by FDM™ 3D-printing technique.

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OSTEOCONDUCTIVE BIOCERAMICS BASED ON CALCIUM PYROPHOSPHATE

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Keywords: bioceramics, calcium pyrophosphate, 3D-printing, stereolithography, osteoconductivity

INTRODUCTION

Modern regenerative medicine requires the creation of new materials for bone implantation. The most important properties of such materials are biocompatibility, resorbability, and osteoconductivity. When implanted at the damaged area of bone, the material should be gradually dissolved and replaced with the growing native bone tissue. Being dissolved, such material serves as the source of elements required for the bone formation. Calcium phosphates with the $0,5 \leq \text{Ca} / \text{P} \leq 1,67$ ratio are biocompatible, and their resorption ability increases with a decrease in the Ca / P ratio; therefore, calcium pyrophosphate (CPP) with a ratio of Ca / P = 1 is especially promising.

Osteoconductivity of material for implantation is the ability to provide the proliferation of blood vessels and nerves into the implant. Porous materials with porosity above 60% and pore size not less than 100 μm demonstrate good osteoconductive properties. Creation of porous ceramic materials with high penetration is possible by means of rapid prototyping techniques. Stereolithography is one of the most universal and perspective methods, in which 3D-object is created using photopolymerization of special suspensions.

In order to obtain ceramic material, it is necessary to use suspensions containing powder of the demanded phase composition and a light-cured monomer. However, the printing quality of such suspensions with ordinary white inorganic powders is very poor due to the light scattering at the powder particles. Addition of colorants

allows increase in the printing resolution; however, calcium phosphates obtained by heat treatment ($T < 900^{\circ}\text{C}$) of amorphous calcium phosphate synthesized from solutions of phosphoric acid and calcium acetate, are inherently grey due to the presence of residual carbon.

The aim of our research was to create macroporous calcium phosphate bioresorbable ceramic materials for bone implants with the pre-defined architecture using stereolithography for molding.

This work was divided into several steps. First, we developed a method to synthesize colored powders of calcium pyrophosphate and studied the product properties; second, we obtained and characterized the suspensions for stereolithographic printing containing those powders and light-cured monomer; third, we examined the printing resolution; then, we obtained the “polymer - calcium pyrophosphate powder” composite materials; and, finally, we manufactured the samples of macroporous resorbable ceramics via the heat treatment of those composites and characterized the final product properties.

RESULTS AND DISCUSSION

XRD analysis of the powder after synthesis confirmed the formation of X-ray amorphous product. According to thermogravimetric analysis data, the total mass loss (about 30%) on heating that powder from 20 to 1000°C chiefly occurred over the $40\text{--}600^{\circ}\text{C}$ range. According to mass spectrometry data, the mass loss was associated with the elimination of water (up to 200°C) and the release of the decomposition products of ammonium acetate (at higher temperature). The form of nano-sized particles of the obtained powders was close to isometric. The phase composition of the powders after heat treatment at 500 to 900°C was represented of biocompatible β - and γ - phases of calcium pyrophosphate.

Two parameters were varied in the study of the suspensions for further stereolithography application: the temperature ($500, 700, \text{ or } 900^{\circ}\text{C}$) of preliminary heat treatment of the CPP powder and the content of powder (10–40 vol.%) in the slurry. To characterize the properties of the light-sensitive suspensions, they were exposed to different radiation doses through a special mask. The so obtained “polymer-powder” composites were examined by means of light microscopy. The relations between depth and radius of polymerization and the radiation dose were established. Basing on the obtained data, photosensitivity and critical energy of polymerization of the studied suspensions were identified.

Rheological study of suspensions indicated that all slurries with the powder loading of 20-40% exhibited the non-Newtonian (pseudoplastic) flow behaviour. At the higher concentration of the powder in the suspensions, the flow can become dilatant, i.e. the viscosity grow with the increase in shear rate. To provide the homogenization of suspensions when printing, the low viscosity is required for moulding of prefabricated ceramic unit by means of stereolithography.

2–40% of calcium pyrophosphate powder in the prefabricated ceramic unit (“polymer / inorganic powder” composite) provided for the retention of shape and continuity of the ceramic sample after removal of polymer and sintering during the thermal treatment.

Macroporous prefabricated ceramic unit (Figure 1, left) were printed by means of the stereolithography from the suspension containing 10 vol.% of calcium pyrophosphate powder obtained at 700°C. That suspension was selected for printing of prefabricated ceramic unit because of its low viscosity, fairly high depth of polymerization, and the high printing resolution. The structure with the architecture of gyroid was chosen for stereolithographic printing, since it is one of the most promising models for producing osteoconductive materials. Composite macroporous ceramic samples (Figure 1, right) were obtained after the heat treatment of prefabricated ceramic unit. Predetermined architecture was retained after the heat treatment. The porosity of the prepared ceramic material (more than 86%) is sufficient to provide high osteoconductive properties.

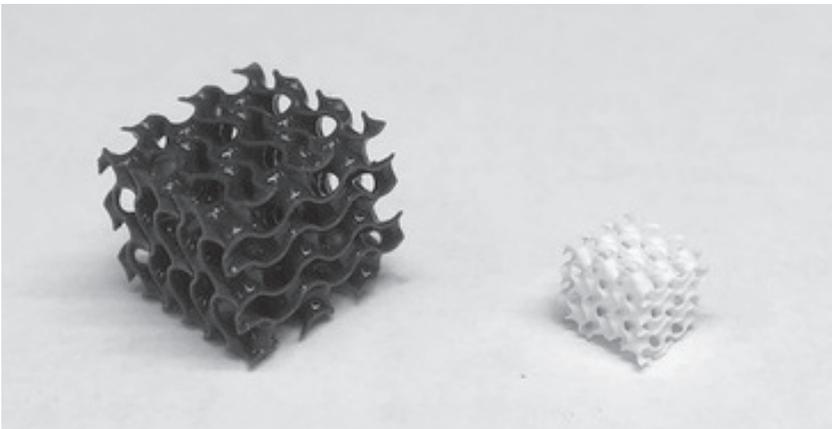


Figure 1: The view of the composite preform obtained by stereolithography from suspension containing 10 vol.% of colored CPP powder (at the left side) and macroporous ceramic sample obtained via heat treatment of that preform (right side)

CONCLUSIONS

The method of amorphous hydrated calcium phosphates synthesis using ion exchange was elaborated. Stable and homogeneous suspensions based on coloured CPP powders and light-cured monomer suitable for stereolithographic printing were obtained. Macroporous ceramic materials with a predetermined architecture were created by means of stereolithographic printing from suspensions based on the coloured CPP powders and the light-curing monomer. The properties of the suspensions (viscosity, photosensitivity, and critical energy of polymerization) were explored. It was shown that the variation of the photosensitivity of suspensions by changing the colour of the CPP powder sufficiently improved the resolution of stereolithographic printing. Ceramic materials with high osteoconductive properties were obtained using stereolithographic printing as a method for moulding the prefabricated ceramic units. Created porous ceramics based on calcium pyrophosphate exhibit good resorbability and osteoconductivity and are suitable for medical applications.

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MACROPOROUS RESORBABLE CERAMICS BASED ON HEAT-TREATED CALCIUM PHOSPHATES WITH LAYERED STRUCTURE

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Keywords: biphasic ceramics, octacalcium phosphate, macroporous biomaterials, slip-casting

INTRODUCTION

Regenerative approach to the treatment of bone tissue is an actual way in modern medicine. Composite bioceramics made of calcium phosphates are the most prospective as bioactive implants for bone grafting. For a long time, synthetic hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (HAp), was extensively used in medicine for restoring bone damages because of its chemical and physical similarity to the inorganic constituent of bone tissue. The main disadvantage of such a material is low resorption (dissolution) rate and weak osteoconductivity. Nowadays, alternative materials are searched for a bone tissue replacement, e.g. biphasic composites derived from calcium pyrophosphate $\text{Ca}_2\text{P}_2\text{O}_7$ (CPP) and tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ (TCP) or TCP/HAp.

Convenient precursors to fabricate such mixtures are layered calcium phosphates, in particular, octacalcium phosphate $\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$ (OCP) and $\text{Ca}_8(\text{HPO}_4)_2 \cdot x\text{R}_x(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$ (intercalated-OCP), in which a hydrophosphate-anion is substituted by a carboxylic acid-anion (R). OCP became the thrust of the study due to its layered structure constructed of alternating apatitic and hydrated layers. Hydrophosphate groups in OCP hydrated layer can be replaced by carboxylate-anions, e.g. succinic- and citric-anions. Such a replacement leads to increasing of Ca/P ratio in substituted phosphate, and makes it possible to obtain biphasic ceramics with variable composition and bioresorption rate. Due to featured layered structure of OCP, OCP-crystals

have a plate-like habitus. If dehydration of the OCP crystals proceeds topotactically while heat treating of the ceramic precursor, one can arrange conserved plate-like particles of the treated OCP-precursors rather tightly, like bricks, in the course of ceramic forming. This might have a beneficial effect on the strength of the ceramics. Additionally, in sense of improvement of osteoconductive properties, macroporous ceramics with tailored pore architecture are to be created.

This work, thus, was aimed at elaboration of the powder precursors derived from heat treated OCP, in order to fabricate biphasic macroporous ceramics (TCP/ CPP, TCP/HAp) with variable Ca/P ratio and phase composition. This scope of the work assumes a search for synthesis conditions of OCP (both "pure" and "intercalated" ones), as well as further study of their thermolysis and the ways to fabricate macroporous osteoconductive ceramics.

RESULTS AND DISCUSSION

Brief description of T-pH conditions of OCP synthesis was done with pH-titration and pH-stating. Properties of as-synthesized samples, as well as thermolysed ones, were characterized by powder X-ray diffraction (powder XRD), simultaneous thermal analysis (STA), infrared spectroscopy (IR) and scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM/EDX). Linear shrinkage of dense ceramics during its sintering was studied by dilatometry and by geometrical measurements.

In this study, substitution of HPO_4^- -anions was done by treatment of β -TCP aqueous suspension in buffer solutions of succinic acid (H_2Suc , $\text{Suc} = \text{OOC}(\text{CH}_2)_2\text{COO}^{2-}$) and citric acid (H_3Cit , $\text{Cit} = \text{C}_3\text{H}_5\text{O}(\text{COO})_3^{3-}$) at definite temperatures and pH-values. The pH-values were chosen according to the area of stability of OCP-phase in T-pH space (Figure 1) constructed from both [1] and our own data.

According to XRD, it was shown that HPO_4^- -anion can be substituted only by succinate-anion to form Suc@OCP . Incorporation of citrate anion in OCP structure didn't occur apparently due to the odd basicity of the corresponding acid. It was also claimed that the synthesis of Cit@OCP (assuming its existence) requires a lot of time (about 10 days). The processes of thermal decomposition of OCP and Suc@OCP were investigated by thermal and X-ray analyzes. It was suggested that intercalated-OCP is more resistant to thermolysis compared to a pure OCP. Decomposition of "intercalated"-OCP occurs more slowly because of simultaneous liberation of CO_2 and H_2O from the lattice. Heat-treated OCP particles keep their plate-like shape and have no crystallized water being heated to 300°C . An intermediate product of

the thermolysis can be described as an apatite-like phase. Ceramics formed from decomposed Suc@OCP have greater shrinkage ($\approx 30\%$, relative density – 90%) and lower porosity compared to the ceramics made from heat treated OCP ($\approx 15\%$, relative density – 75%). To create macroporous ceramics the following techniques were tested: the replica method (impregnation of the PU polymer framework by ceramic slurry), the method of removable additives (molding powder together with burning additives, e.g. polystyrene beads) and slip-casting into the plastic molds with special architecture created by FDM 3D-printing (water-based slips as well as paraffin wax thermoplastic slips were used).

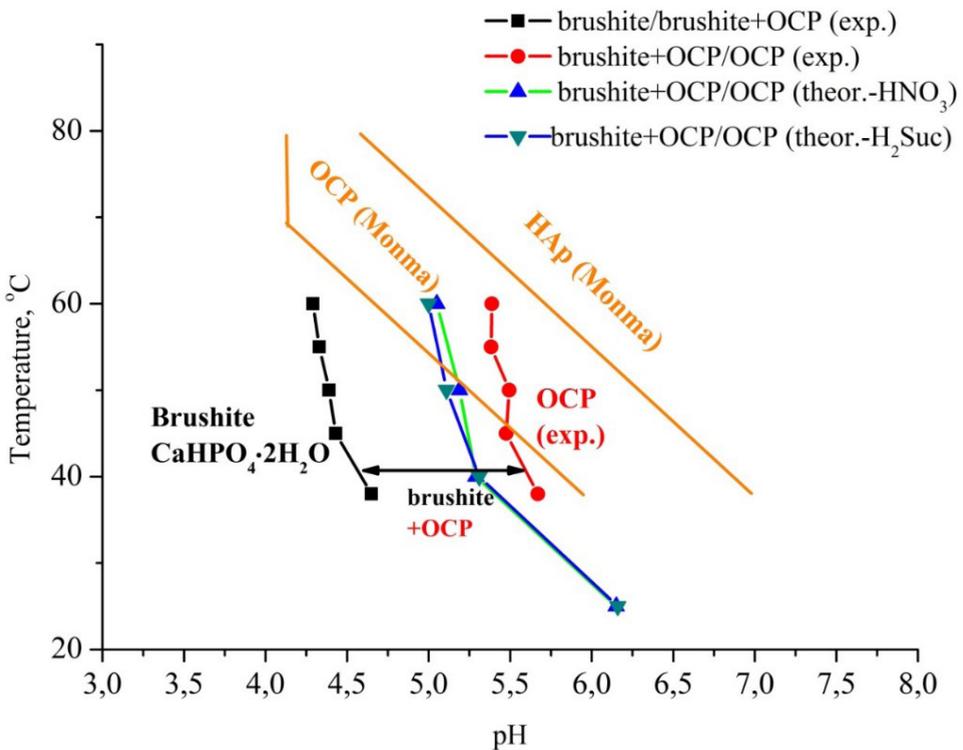


Figure 1: Area of OCP stability in T-pH space (thin solid lines without shapes are related to experimental data on brushite treatment in solutions, according to [1]); our data on hydrolysis of α -TCP in aqueous solutions of nitric and succinic acids are presented as lines with shapes (exp. – our experimental data on pH-titration, theor. – our calculations of ionic equilibria done with HYDRA/MEDUSA and Visual MINTEQ v.3.1).

CONCLUSIONS

Thus, detailed theoretical and experimental studies of ionic equilibria in buffered solution containing brushite or α -TCP were undertaken to determine the area of coexistence of OCP and brushite in T-pH space. These results were used to get the conditions of synthesis of OCP-phases. Various techniques and conditions of solution synthesis have been tested for obtaining of intercalated. The pure product of substitution $\text{Ca}_8(\text{HPO}_4)_{2-x}\text{R}_x(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$ ($\text{R} = \text{Suc}$) with $x = 0,8 \div 0,9$ was acquired by hydrolysis of α -TCP in the succinic buffer, but the ways to control the degree of substitution x have not been found yet. Suc@OCP demonstrates greater resistance to thermolysis compared to pure OCP. The differences in kinetics and mechanism of their decomposition lead to a different micromorphology of intermediate apatitic product, which is stable up to 630°C in the case Suc@OCP. Variation in phase composition of the thermolysis products leads to the fact that the ceramics based on heat-treated Suc@OCP are denser and apparently stronger than ones based on heat-treated pure OCP. It is shown that slip-casting in 3D-printed plastic forms provides the best reproduction of complex macroporous architecture.

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PARTIALLY CROSSLINKED STARCH FILMS CONTAINING CALCIUM PHOSPHATES

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Keywords: starch, calcium phosphates, mineral-polymer films composites.

INTRODUCTION

Starch is a widely common polysaccharide that is used in medicine for tablets production, as component of gels, in the treatment of burns [1,2]. Starch possesses film-forming properties but the swelling rate of starch films is too large. To decrease swelling rate some reagents as citric acid, glyoxal, glutaraldehyd, calcium, magnesium, iron salts are used to link the starch polymer chains [3].

The incorporation of different calcium phosphates into the starch matrix opens new perspectives for the application of composite starch-calcium phosphate materials in regenerative medicine.

RESULTS AND DISCUSSION

To prepare starch films the starch 2 or 4% water gels were used. The citric acid and glyoxal water solution were chosen as crosslinking agents.

The calcium phosphates were synthesized *in situ* in starch water gels using initial salts of calcium nitrate and ammonia dihydrophosphate varying both the Ca/P relation and the acidity as well. It was revealed by XRD analysis that dicalcium phosphate dihydrate

is formed at $\text{Ca/P} = 1$ and $\text{pH} = 5.5$. Precipitated hydroxyapatite (PHA) was detected when $\text{Ca/P} = 1.5$ and $\text{pH} = 7$. The same calcium phosphate was determined to form at $\text{Ca/P} = 1.67$ and $\text{pH} = 9$.

Composite films microstructure was studied by TEM and was shown to depend on the calcium phosphate form (Figure 1). Dry composite films are brittle: elongation was up to 1.5% and ultimate breaking strength was about 20 MPa. After immersion of the starch film into the water for 5 sec swelling degree was detected to be 20%. The elasticity of wet film increased and elongation rises up to 10% and Young's modulus decreased by 10 times.

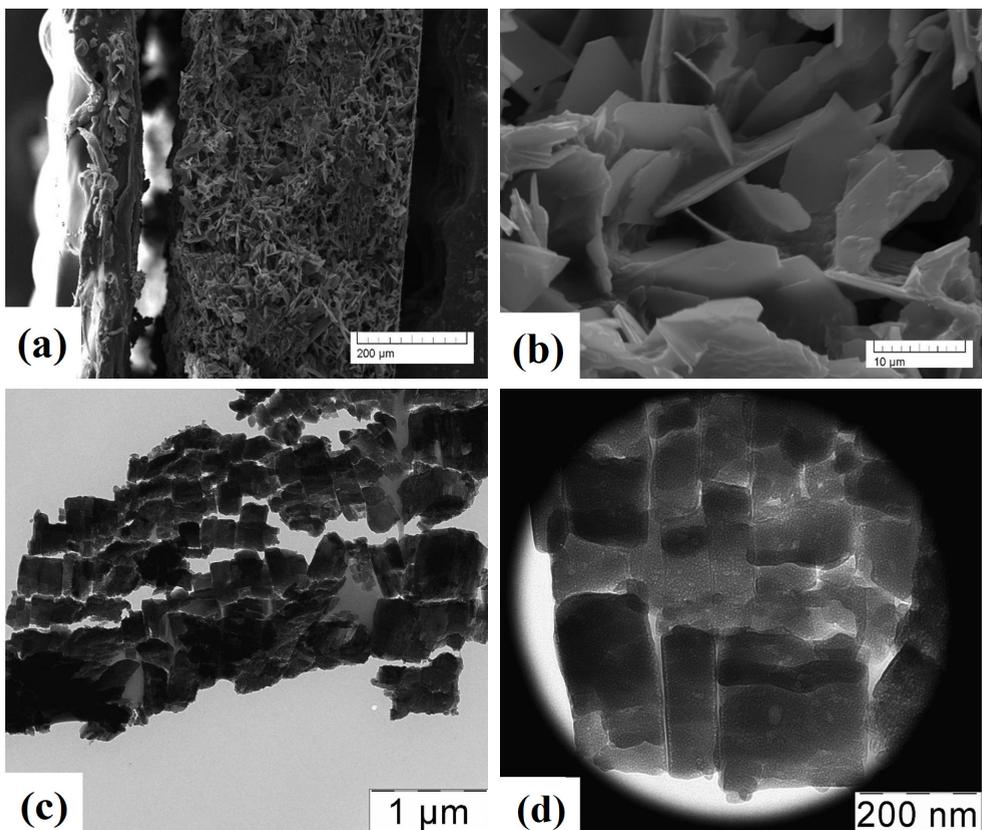


Figure 1: (a, b) SEM and (c, d) TEM micrographs of composite starch film with DCPD.

The samples obtained with citric acid were revealed to possess higher mechanical properties when comparing with samples prepared with glyoxal.

The cytotoxicity of starch-based materials was studied on the fibroblasts NCTC L929 according to the standard R ISO 10993. To investigate the adhesive characteristics of the materials and their impact on the cells activity the primary culture of human fibroblasts was used.

This work was financially supported by Presidium RAS, Program N° I.I.P.

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ALPHA-TRICALCIUM PHOSPHATE BASED BRUSHITE CEMENT FOR OSTEOPLASTICS

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Keywords: calcium phosphates, brushite bone cements, microstructure evaluation.

INTRODUCTION

Calcium phosphate cements (CPCs) are used to fill bone tissue defects arising upon surgery, diseases, or injuries [1, 2]. CPC have a simple protocol of their preparation and usage, their curing is situated under physiological conditions, and they demonstrate osteoconductive properties. The cements comprise a solid (powder) and liquid components, their mixing results in setting and hardening of the cement paste due to acid-base chemical reactions occurring in the paste, and leads to bonding of the paste to a strong monolith cement stone. The reaction of chemical bonding gives rise to a set of interpenetrating crystals of a targeted phase - apatite or dicalcium phosphate dihydrate (brushite structure), depending on formulation. The strength of CPC is provided by both van der Waals forces in point contacts of contiguous crystals of aforementioned phases and ionic-covalent bonds in extended phase contacts of the crystals. The bioresorption rate of brushite cements (BCs) is close to growth rate of bone *de novo*, though the level of compressive strength of BCs is too low – it does not exceed 10 MPa. Another drawback of BCs consists in significant acidification of surrounding tissue in the course of BCs bioresorption.

Thus, the aim of present study was to formulate a new BC with improved strength and pH level of its aqueous extract close to that one of body fluids.

RESULTS AND DISCUSSION

New formulation of bone cement based on alpha-tricalcium phosphate (α -TCP) with dense ceramic granules of carbonated hydroxyapatite (CHA) was developed. CHA granules were made by crushing of dense CHA ceramics [3]. The 30% water solution of magnesium dihydrophosphate was used as a hardening liquid. Setting time of such cement is too low for its application; to solve the problem some reagents (among them, sodium hexametaphosphate and sodium citrate) were tested as setting retardants. The setting time of BCs modified with sodium hexametaphosphate was extended to 8-10 min. Addition of CHA granules decreased BCs acidity and, at the same time, increased the mechanical strength of the cements. It was found that a surplus of 10% of CHA granules made it possible to obtain the BCs with pH 6,9-7,2 of their aqueous extract and compressive strength of about 20 MPa. It worth noting that such a high value of compressive strength for BCs was reached for the first time.

The microstructure of the cements under study changed significantly during first 3 days after mixing (Figure 1) while the phase composition remains unchanged. This fact implies recrystallization of brushite phase leading to better bonding of neighboring crystals, and, hence, a correlation between cement microstructure and the compressive strength occurs. The microstructure of as-mixed cement is highly heterogeneous (Figure 1a) and the compressive strength appears to be not more than 2 MPa. After 24 hours cement structure became more homogeneous (Figure 1b). After 96 hours of hardening, plate-like crystals covered with a kind of amorphous substance (Figure 1c) are observed, and the strength grows up.

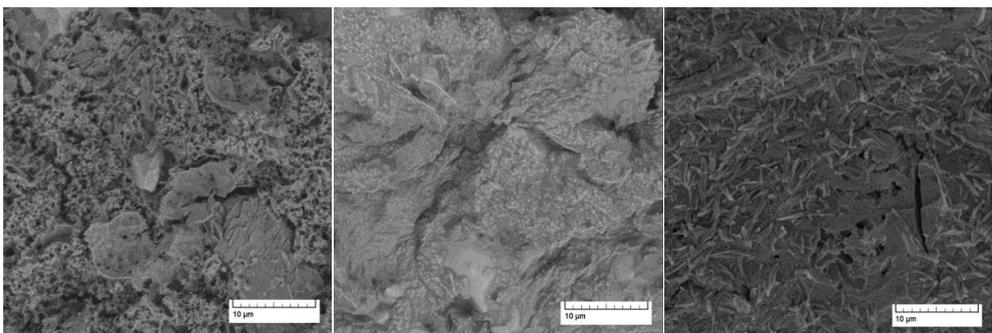


Figure 1: Evolution of the cement microstructure during setting and hardening: a – 10 min; b – 24 hours; c – 96 hours after mixing.

Magnesium phosphate was not detected by XRD despite of its content in the cement is of about 12 wt.%. This fact allows us to conclude that the amorphous substance consists of magnesium phosphate. EDX analysis of intercrystalline regions confirmed this assumption.

After 96 hours of hardening, the compressive strength of the cement exceeds the value of 17 MPa, being 1,5-2 times higher compared to known formulations of brushite cements.

CONCLUSIONS

Thus, the developed formulation seems to be perspective for osteoplastic surgery accounting for its acidity and compressive strength level.

This work was financially supported by RFBR, grant N° 15-29-04871-ofi_m.

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SMART SCAFFOLDS FOR OSTEOPOROSIS TREATMENT

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Keywords: smart scaffold, osteoporosis, biofabrication, osteoblast-osteoclast coupling

INTRODUCTION

Osteoporosis is a well known, worldwide spread disease with a rapidly growing incidence as the population ages; it results in bone loss and deterioration and in a decreased bone strength involving an increase in the risk of fractures. This disease has a very high frequency in people over 50 and it has been calculated that 1 in 5 men and 1 in 3 women over 50 will experience an osteoporotic fracture in their lifetime [1], see Figure 1.



Figure 1: Osteoporosis occurrence in women over 50

The main clinical consequences of this disease are bone fractures, which are associated with significant morbidity and mortality. Antiresorptive agents such as bisphosphonates are mainstays of the therapy for osteoporosis and currently four of these agents have received FDA approval for clinical treatment. The perfect solution to treat osteoporosis is still not within grasp and recently attention was drawn to the negative outcome of some drugs clinically used to treat osteoporosis [2]. In the frame of the ERC-consolidator grant BOOST, a scaffold purposely developed for osteoporosis treatment will be developed.

In the present work, healthy and early osteoporotic bone geometries will be obtained from tomographic scans of human bone tissues discarded from surgery on healthy and early-stage osteoporotic patients. Smart scaffolds will be biofabricated by means of a purposely developed multimaterial platform which will combine different rapid prototyping techniques. To manufacture the scaffolds collagen will be used as a matrix and mesoporous bioactive glass as reinforcing and bioactive phase. The fabricated scaffolds will then be tested in suitable bioreactors by means of a co-culture of osteoblasts and osteoclasts in order to codify the influence of both chemical and topographical stimuli on the osteoblast-osteoclast coupling.

RESULTS AND DISCUSSION

Ethical approval to retrieve human healthy and osteoporotic bone had been obtained at the Istituto Ortopedico Rizzoli. Protocols for the co-culture of osteoblast and osteoclast are currently under development at Istituto Ortopedico Rizzoli and Università Politecnica delle Marche.

Mesoporous glasses based on the SiO₂-CaO system containing different doping ions (Cu²⁺, Sr²⁺) have been prepared both by an ultra-sound assisted base-catalyzed sol-gel method and by an aerosol-based spray-drying process, in order to control both the particle size and their morphology.

The structural and morphological features have been investigated by TEM and FE-SEM coupled to EDS, N₂ adsorption-desorption, XPS as well as their ability to form hydroxyapatite in vitro. The release profiles of the therapeutic ions have been measured by inductively coupled plasma-atomic emission spectrometry. FESEM image of spray-dried mesoporous glasses shows micro-sized spherical morphology, with a size ranging between 500 nm and 5 μm without the formation of aggregates (Figure 2, left). The particles prepared by ultra-sonication showed spheroidal nanoparticles with size of ca 100 nm, with a slight tendency to aggregate (Figure 2, right). The EDS

quantitative analysis revealed element ratios very close to the theoretical ones.

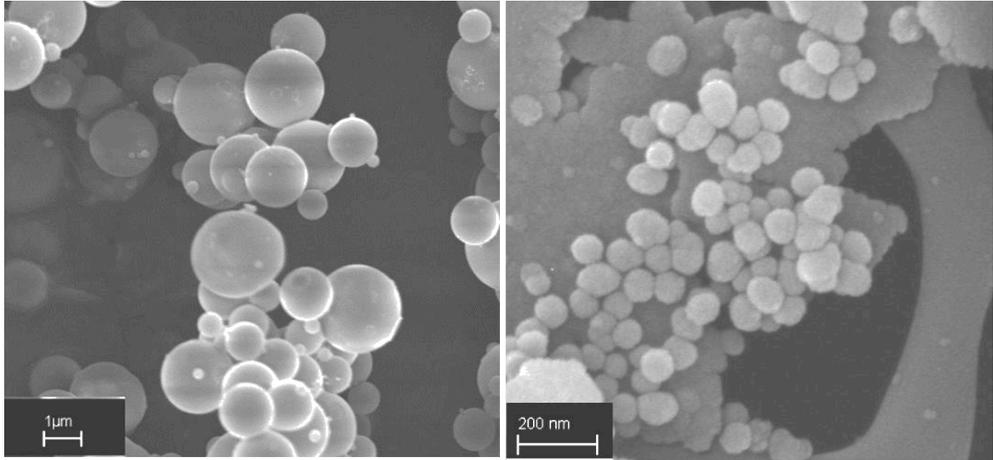


Figure 2: FE-SEM image of SiO₂-CaO containing Cu²⁺ prepared by spray drying (left) and by ultrasound-assisted synthesis (right).

OUTLOOK

The proposed approach will allow to codify how biomaterial chemistry and topography at the macro-, micro- and nano-scale influence the multifaceted coupling process of bone resorption and formation, with special emphasis on the cell cross-talk between osteoclasts and osteoblasts.

ACKNOWLEDGEMENT

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MICROSCAFFOLDS WITH SELECTED PERMEABILITY FOR REGENERATIVE MEDICINE

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Keywords: polymethyl methacrylate, regenerative medicine, microencapsulation, porosity

INTRODUCTION

Poly(methyl methacrylate) (PMMA) is a biocompatible and non-biodegradable polymer currently widely used as biomedical material [1]. Recently its application as carrier to encapsulate live mammalian cells for treatment of a variety of diseases is of particular interest [2].

The goal of this work is to set up the suitable microencapsulation conditions and process parameters in order to achieve a standardized protocol for the encapsulation of viable cell into PMMA beads by “High efficiency vibrational technology” giving reproducible results. The goal is to obtain microcapsule with suitable surface porosity in order to ensure cells viability.

PMMA microcapsules were made using Encapsulator B-395 Pro (BUCHI GmBH). Final set up provided PMMA 10% w/w solution in CH₂Cl₂ extruded through concentric nozzle (400 and 200 μm) under imposed vibration for breaking down the laminar jet into equal size droplets. The droplets were recovered into a biphasic solution made of organic solvent (dodecane) and phosphate buffer saline (PBS), polyvinyl-alcohol 2% (PVA) and Pluronic-127 2% w/w aqueous solution to allow the polymer precipitation and the microcapsules formation. Polymer concentrations tested were between 5 and 15% w/w; polymer to aqueous phases ratios was tested between 1:10 and 1:50. In order to achieve control of surface porosity, the microcapsules were prepared following the results of a DoE screening protocol on additives and organic phase variables. .

Process parameters such as vibration (frequency and amplitude), electrostatic dispersion (voltage), light intensity of the stroboscope lamp, syringe pump (pump speed and calibration) and stirring/ shaking were optimized in order to obtain homogeneous and reproducible batches of beads.

The microcapsules have been characterized for their morphology (SEM) and cytocompatibility by MTT test, mechanical and permeability properties and osmotic resistance.

RESULTS AND DISCUSSION

The optimized process conditions were polymer concentration 10%, polymer : aqueous phases ratio 1:50, frequency vibration 2000Hz and 350 V, pump speed 10.5 ml/min.

Microcapsules with narrow size distribution in the range 500-800 μm have been obtained with yields process about 60%. The Microcapsules were stable to mechanical and osmotic stresses.

Permeability test, using pullulans standard, showed that the process conditions significantly affect microcapsule membrane permeability. The membrane of microcapsules prepared by the optimized process conditions resulted to be permeable to molecules of 180 Da and 5900 Da Mw, while preventing diffusion to molecules of 11.100 Da Mw..

MTT test results showed good microcapsules cytocompatibility for PMMA: cells ratio lower than 10 mg/20.000 cells. Morphology characterization by SEM showed smooth microcapsules surface with regular spherical shape and homogeneous porosity.

CONCLUSIONS

The microencapsulation process confirmed to be reproducible and to control microcapsule formation. Controlled porosity permits the passage of selective molecules with defined Mws. Future perspectives involve set up of manufacturing process under sterile conditions and process scale-up.

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3D PRINTED CHITOSAN-BASED HYDROGELS IMPROVING FIBROBLAST GROWTH

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Keywords: Chitosan, hydrogels, 3D printing, fibroblast cells

INTRODUCTION

The general aging of population and the increase in the prevalence of chronic diseases, injuries or traumas lead to an augmented demand of drugs and biological substitutes that could restore, maintain or improve tissue function [1]. The main target of tissue engineering is the identification and application of adequate materials for the design and production of supportive structures, generally defined as scaffolds, that possess desirable properties for promoting cell adhesion, proliferation and differentiation [2]. Among natural polymers possessing interesting features, thanks to its resemblance to extracellular matrix and, in general, for its better cytocompatibility, chitosan, a β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, is widely used. It possesses a good combination of biocompatibility and biodegradability, it is not toxic and not expensive, and it can be molded into any desired shape, thus making it suitable for many applications [3]. Since the performances of scaffolds depend both on the characteristics of their components and on their structural architecture, in this work we propose the preparation

of 3D printed chitosan hydrogels for tissue regeneration applications. Chitosan scaffolds are prepared by deposition of the solution in a cryogenic chamber followed by direct transfer into a coagulation bath. This is the direct transposition and automation of the freeze-gelation method described in the paper by Elviri et al. [4] and associates the advantages deriving from a chitosan-based solution to the technical advantages of 3D printing. A technical description of the automation system and the parameters that determine a satisfactory deposition are reported. These include, among others, the role of the viscosity of the starting chitosan solution and the effect of temperature on the success of deposition and scaffold appearance. Resulting scaffolds were characterized in terms of morphology and porosity and, finally, in vitro culture studies are reported to compare 3D printed scaffolds with their homologous produced by the traditional approach [4]. Scaffolds were produced starting from chitosan solutions and from chitosan solutions containing raffinose according to the protocol described by Bettini et al [5] and their performances were compared by performing cell cultures.

RESULTS AND DISCUSSION

A chitosan and a chitosan containing raffinose solution were tested to produce scaffolds of different thickness, depending on the number of layers desired (from 5 to 20 layers), with pores of the expected diameter of 400 μm .

The homogeneity and viscosity of the chitosan solution play a fundamental role on the printability and final structural quality of the 3D scaffold. By performing different printing tests the optimal viscosity of the chitosan solution ranged between 8000 and 25000 cP. The temperature of the CH at the extrusion point increased in a non-linear manner with the number of layers (from -18 ° C for the first layer to -8°C at the layer n.26). The chitosan solution extruded immediately freezes once in contact with the surface of the underlying layer allowing the proper manufacture of the scaffold. The optimal temperatures allowing to obtain a controlled CH structure range between -10 and -5 ° C for the point of extrusion and, between -13 and -9 ° C for the middle and the tail point of the filament.

ESEM analysis carried out on scaffolds in a fully hydrated state allowed to verify the excellent agreement (mean change 8%; RSD 15%) between the experimental and theoretical structure designed with the CAD program. The SEM images allowed to verify the interconnectivity and the orientation of the micro-pores, that can be beneficial to direct migration and cellular biosynthesis. In a further step the capabilities of the

printed scaffold to retain water and rehydrate were tested. Following dehydration in oven at 30 °C for 3 hours scaffolds have suffered a homogeneous and significant loss of weight (from 67±4% to 79±3%) not affected by the number of layers. The rehydration step, showed that the scaffolds from 5 to 15 layers, rehydrated up to 75% in only 15 minutes and did not change over 24h.

The 3D-printed scaffolds prepared from chitosan and chitosan enriched with raffinose were thus characterized for the interaction with cells and their capacity to allow cell growth. The results were compared with those obtained on traditional chitosan scaffolds, prepared as described by Elviri et al. [4]. For this purpose, in vitro experiments with human fibroblasts were performed. Cells seeded on both plain and 3D chitosan scaffolds displayed a significant and similar increase in number from day 7 to 14 (+2 fold and +1.5 fold for chitosan and 3D chitosan, respectively; $p < 0.01$ vs day 7); at day 14, fibroblasts onto reticulated scaffold did not further proliferate, but cell number was maintained similar to day 7; conversely, cells onto plain scaffolds decreased to values similar to day 7 ($1.03 \pm 0.21 \times 10^4$ compared to $0.80 \pm 0.13 \times 10^4$; $p = ns$), indicating a lowering in their metabolic activity, probably because of cell death. Conversely, both chitosan-raffinose scaffold types allowed a time-dependent cell growth from 7 to 28 days, that was more pronounced for reticulated scaffolds. Comparing fibroblasts number at each time point of analysis, we observed that, at 7 day of culture, cell number was roughly similar on all scaffolds, while after 14 days a significant higher number of cells was detected on printed chitosan-raffinose scaffold (on average +1.43 fold increase compared to others; $p < 0.01$); such difference was still appreciable after 28 days from plating (on average +1.6 fold increase compared to others; $p < 0.05$).

Finally, we measured the proliferation efficiency of fibroblasts allowed to grown onto scaffolds for 14 days and subsequently detached and re-seeded on regular well plates. As control, we considered an equal number of cells detached from a regular well plate and undergoing the same culture conditions. Only cells detached from reticulated chitosan-raffinose scaffolds reached a number similar to control ($3.85 \pm 0.04 \times 10^4$ compared to $4.12 \pm 0.5 \times 10^4$; $p = ns$), while on all other scaffolds cell number was significantly lower.

CONCLUSIONS AND/OR OUTLOOK

The results obtained in this study clearly evidenced that the scaffolds made of chitosan and raffinose with the 3D architecture appear to be the more suitable supportive template

for fibroblasts growth overtime, with promising tissues engineering applications. By using 3D printing chitosan scaffolds could be designed in combination with a variety of polysaccharides or active compounds with selected and reproducible space distribution, providing applications as active wound dressing or tissue regeneration.

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MECHANICAL CHARACTERISATION OF SILICA-BASED HYBRID MATERIALS FOR TISSUE REGENERATION

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Keywords: hybrid, sol-gel, mechanical characterisation

INTRODUCTION

Hybrid materials offer the possibility of combining the bioactive properties of an inorganic component with toughening via the organic constituent. As nanoscale interpenetrating co-networks [1], they can do this without suffering, for instance, the masking effect of a polymer matrix in a composite material. However, their structure poses a problem for mechanical characterisation. Techniques applicable to polymeric materials are not compatible with ceramics, and *vice versa*. Furthermore, a hybrid is not a simple mixture and cannot be understood as a combination of properties in the same way as a composite. Here, an attempt is made to characterise more fully the mechanical behaviour of a novel silica-based hybrid, encompassing tensile, compressive, time dependent and toughness testing.

The ability to fabricate tissue scaffolds with sufficient and stable mechanical properties is an important step, crucial to the success of a scaffold implant. In general, there is a requirement to match the properties to the tissue that will surround the implant and this means that different applications result in vastly different property requirements from the scaffold. For example, intervertebral disc (IVD) replacement requires a radial gradation of stiffness. Complete IVD replacement represents an ongoing challenge in tissue engineering (where the conventional treatment of spinal fusion does not retain the biomechanics of the spine). Therefore, it is desirable to be able to tune the mechanical properties of the hybrid materials.

One route to achieving this is an understanding of the effect of the synthesis procedure

(extent of polymerization, gelation time) and composition on the resultant mechanical properties. There is as yet no standard protocol for the mechanical testing of hybrids, so the simultaneous development of a set of characterisation procedures is vital in order to compare them reliably to the alternative materials (in this case polymers, ceramics and composite materials).

RESULTS AND DISCUSSION

Here, silica-based hybrid materials were produced via the sol-gel process to obtain a range of silica contents (2 – 45 wt%). The upper limit to the range is limited by the ability to fabricate the hybrids without shattering (above 50 wt% silica the hybrids do not maintain mechanical integrity on fully drying). Figure 1 shows the failure stresses in compression for four compositions. Over the whole composition range, these hybrids show promising compressive strength and ability to deform significantly in compression (Figure 2); for instance at 20 wt% silica content they reach above 50 % strain at failure. The strain at failure decreases with increasing inorganic content, consistent with the lower flexibility expected of glassy materials, whereas there is a maximum in the ultimate compressive strength for an intermediate inorganic content. The stress-strain curves show increasing apparent stiffness with the applied stress.

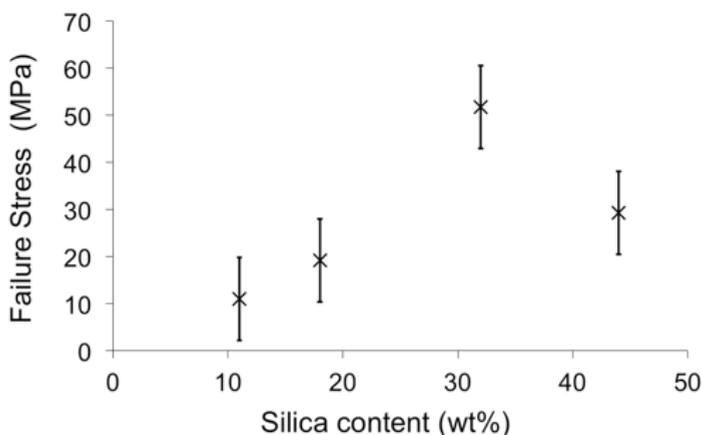


Figure 1: Failure stress under uniaxial compression for four hybrid compositions, in the form of cylinders (aspect ratio 2).

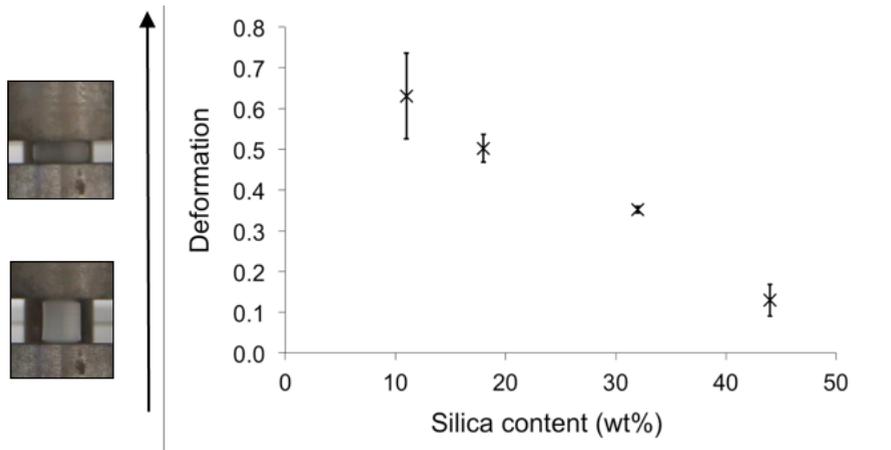


Figure 2: Strain values at the maximum compressive stress, showing an expected decrease with increasing silica content. Even at 44 wt% silica, the hybrid reaches above 10 % deformation.

Preliminary results from cyclic tests up to 60 % of the failure strain, over 10 cycles with one minute dwell, have indicated that the deformation in this region is fully recoverable. Hysteresis occurs between the loading and unloading cycles, demonstrating the viscoelastic behaviour of the material.

Inclusion of polymer has previously been shown to increase the compressive strength of the material compared to (brittle) glass scaffolds, for example in gelatin-silica hybrids [2]. However, the elastomeric behaviour that has been observed in this hybrid has not been seen previously in similar silica-based hybrid materials.

OUTLOOK

Further testing is being carried out to determine the dynamic and fatigue behaviour (DMA, cyclic testing) and fracture toughness. The latter is particularly challenging due to the hybrid nature of the material, not traditionally brittle despite the glass content, and also crucial to the final goal of using the hybrid in a tissue engineering context. Significant shrinkage of the material during the drying process means fabricating samples of the required dimension for testing is also not straightforward.

The ability to subtly vary the stiffness, and the range of failure stress and strain values obtained, show promise for application in entire disc replacement of intervertebral discs (IVDs). This would represent a big improvement in an area currently lacking in satisfying solutions.

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SOL-GEL SILICA DOPED WITH POLYMER TAILORED AGNP FOR TISSUE REGENERATION

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Keywords: silver nanoparticles, PVA, PVP, silicates, sol-gel

INTRODUCTION

Hybrid biomaterials built of silicates and silver nanoparticles (AgNP) are multifunctional materials desirable for infection treatment and prevention due to the combined properties of each component. The insertion of nano-silver in the glass matrix creates new biomaterials ideal for healing and regeneration processes. In order to obtain stabilized AgNPs, surface modification of the nanoparticles is needed. Polyvinylpyrrolidone (PVP) and polyvinyl alcohol (PVA) are popular coating agents in AgNP synthesis because they have excellent compatibility with nano-silver, without any modification of the nanoparticles surface. They are used as size controlling and stabilizing agents because they inhibit the growth of silver nanoparticles by surrounding their surface. In recent studies, blends of PVA and PVP coatings have been reported as successful stabilizing agents.

Silver nanoparticles have been successfully synthesised by using different ratios between two stabilising agents: PVA and PVP dissolved in distilled water. The final AgNP solutions contained different blends of the polymeric solutions for different concentrations of Ag. The polymers acted as stabilizing agents, due to their high affinity towards silver nanoparticle surface. The polymer-stabilized AgNPs were introduced in sol-gel prepared SiO₂ glass systems and characterized by UV-vis spectroscopy, X-ray diffraction, Fourier-transformed infrared spectroscopy, and transmission electron microscopy. Afterwards, they were investigated *in vitro* by antibacterial assay and cell culture viability tests.

RESULTS AND DISCUSSION

Uv-vis spectroscopy and TEM analysis have demonstrated the successful synthesis of silver nanoparticles stabilized in aqueous media by PVP and PVA. The specific SPR peaks for AgNPs (around ~ 400 nm) were detected around 425 nm for all samples. This shift can be explained by the presence of the polymer coat surrounding the AgNPs, which has a different refractive index than the solvent. TEM images showed even distribution of the AgNP throughout the solution and also good particle size distribution with average particle diameter between 5-10 nm. TEM images for the samples with higher concentration of silver showed that AgNP tend to be entrapped inside the polymer matrix whereas for equal parts of PVA and PVP the AgNPs are not confined by the polymers.

The AgNPs stabilized by PVA and PVP for low silver concentration have been inserted into silica glasses by quick-set sol-gel method. Also, silver nitrate (previously diluted in distilled water) was introduced into silica glass in order to establish differences between the polymer-stabilized AgNP and bare nano-silver. XRD and FTIR results suggest that the polymers and silver did not change the structure of the glass matrix, as no changes could be detected in the obtained spectra. These results indicate successful insertion of the AgNP into the glass matrix. TEM images (Figure 1.) show that AgNPs are evenly distributed all over the glass matrix.

In vitro tests displayed the excellent antibacterial effect of silver nanoparticles on both gram positive (*S. Aureus*) and gram negative (*P. Aeruginosa*) antibiotic-resistant bacteria strains. Cell viability results suggest that silicates doped with AgNP did not negatively affect the viability of healthy epithelial cells (tested on human retinal pigment epithelial cell line D407).

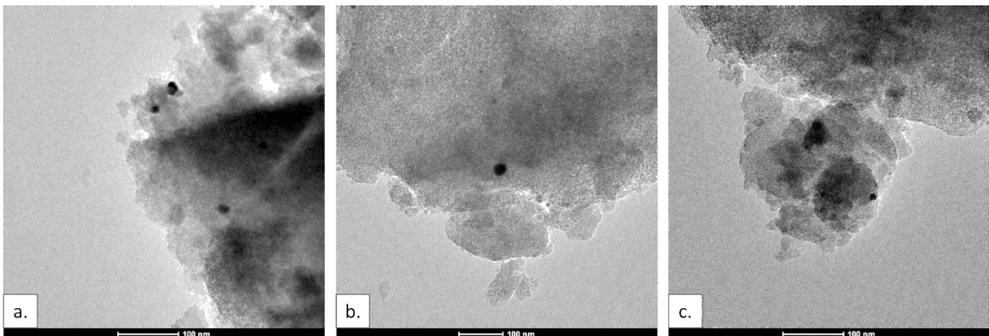


Figure 1. Silicates doped with AgNPs: a.Si-AgNO₃; b. Si-AgPVA; c. Si-AgPVP.

CONCLUSIONS AND/OR OUTLOOK

Successful synthesis of silver nanoparticles in aqueous media has been achieved using different ratios of polymers as stabilizers. The AgNPs were successfully inserted into silica glass matrix and *in vitro* studies showed promising results for their future use and biomaterials.

ACKNOWLEDGEMENTS:

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SYNTHESIS OF POLOXAMER-BASED THERMO-RESPONSIVE POLYMER AND ITS CYTOTOXICITY ACTIVITY

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Keywords: Polymers, Hydrogels, ATRP

INTRODUCTION

Smart polymers are used in various biomedical fields. Smart polymers or stimuli-responsive polymers actively react to small signs and changes in the surrounding environments such as temperature, ultrasounds, light and mechanical stress by showing physical or chemical changes in their behavior. Thermo-responsive polymers are temperature-sensitive thus, change their microstructural features in response to changes in temperature [1].

RESULTS AND DISCUSSION

Synthesis

Using Atom transfer radical polymerization method (ATRP) [2], a poloxamer-based conjugate was synthesized. First, Poloxamer 407 I was modified by 2-chloropropionyl chloride in CH_2Cl_2 . The product 2 was then reacted with 2-(dimethylamino)ethyl methacrylate (DMAEMA) in the presence of $\text{CuBr}/2,2'$ -bipyridyl complex in a 1:200 ratio (Poloxamer-Cl : DMAEMA) under an inert atmosphere of nitrogen.

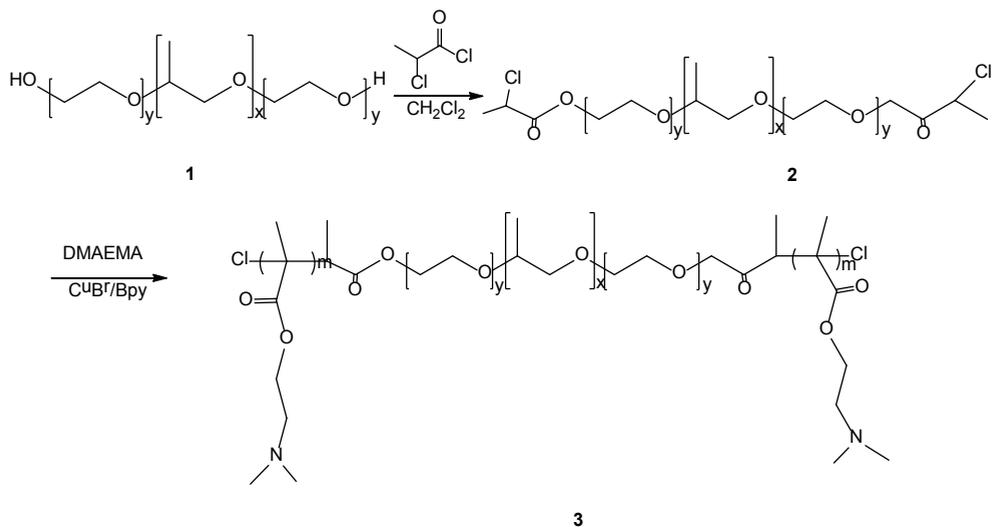


Figure 1. Synthetic scheme of Poloxamer 407-p(DMAEMA)

In vitro toxicity

The polymer was used to condition cell culture media. This was done by soaking it in the media at various time intervals (from 1 hour until 24 hours) and then assaying the media for its impact on cultured cells. At the time points of interest, the polymers were removed from the media, which was then frozen. In vitro cytotoxicity assay was used. Human fibroblasts were seeded in a 96-well culture plate in a humidified 5% CO_2 atmosphere. Cells were serum-starved for 24 hours and then incubated with the polymer-conditioned media for a further 24 hours. After 24 hours, MTT assay test was performed and the absorbance at 570 nm was measured with a plate reader at time intervals of 12 hours, 24 hours and 48 hours. MTT test revealed improved fibroblast growth in the presence of tested polymers and no toxic effects were recorded.

CONCLUSIONS

By means of ATRP Poloxamer 407-p(DMAEMA) was obtained. Results from cell proliferation assay suggest that the DMAEMA has no toxic effects on cells.

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HYDROLYSIS STUDY OF POROUS LACTONE-BASED COPOLYMERS

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Keywords: Polymer, ScCO₂ foaming, lactide, ε-caprolactone, scaffold

INTRODUCTION

Autograft is the golden standard in contemporary skull reconstruction. However, the use of it is not always possible and therefore synthetic materials are needed. Titanium, acrylates, and hydroxyapatite are currently widely used in reconstructive processes. All of the currently used materials have high rates of clinical success, however they have also material specific disadvantages [2]. To overcome these disadvantages, resorbable polymers have been studied. [1]

In this study, the aim was to develop resorbable porous materials prepared by supercritical carbon dioxide foaming (ScCO₂) to be used as a scaffold in bone tissue engineering. ScCO₂ foaming enables preparation of interconnected and highly porous structures without harmful solvents or high temperatures. Hydrolysis behaviour of three different self-synthesised lactone-based polymers and one commercial copolymer was studied and the effect of porosity on degradation was evaluated.

MATERIALS AND METHODS

Commercial copolymer poly(L-lactide-co-ε-caprolactone) (monomer ratio 70/30) was purchased from Corbion. Polymerisation of copolymers was done as bulk polymerisation at 160 °C for 4 hours. Polyethyleneglycol (PEG, 20 000g/mol, 0.04 mol-%) was used as co-initiator, stannous 2-ethylhexanoate (0.05 mol-%) was used as initiator and L-lactide (LLA), DL-lactide (DLLA) and ε-caprolactone (CL) were used as comonomers. After polymerisation, polymers were purified by dissolving in

dichloromethane and precipitation in ethanol. Cylindrical samples (diameter 5mm, height 2 mm) were prepared by compression molding and porogenized by ScCO₂ foaming. Porogenization had not significant effect on the molecular weight. Polymers, the comonomer ratios and number average molecular weights after porogenization are presented in Table 1.

Abbreviation	CL (mol ratio)	LA (mol ratio)	Lactide (L or DL)	Co-initiator PEG	Molecular weight (Mn)
C	30	70	L	no	115 000
P1	30	70	L	yes	48 000
P2	30	70	DL	yes	90 000
P3	15	85	DL	yes	100 000

Table 1: Used polymers, their comonomer molar ratios and molecular weight after porogenization. C is commercial and P1, P2 and P3 self-synthesised.

Thermal properties of the prepared polymers were measured by differential scanning calorimetry (DSC). The analysis was performed in the temperature range of -50 °C to 200 °C at heating rate of 10 °C/min. The melting points were analysed from the first heating scan and glass transitions were determined from the second heating scan. Hydrolysis study for porous and bulk samples was done in Sørensen buffer solution (pH 7.4), which was prepared according to ISO 15814 (Implants for surgery – copolymers and blends based in polylactide – in vitro degradation testing). During the hydrolysis, the mass and molecular weight of the samples were monitored. The molecular weights of oligomer species were determined by room temperature size-exclusion chromatography (SEC).

RESULTS AND DISCUSSION

According to DSC analysis, polymers containing L-lactide were partially crystalline, whereas polymers containing DL-lactide were amorphous. For polymers C and P1, the mass of samples remained constant until week 14 and for polymers P2 and P3 until week 8. As expected, the mass of the amorphous polymers decreased faster. Molecular weight of the samples during the hydrolysis are presented in Figure 1. Even though there was not change in the mass of the samples during 8 weeks, the molecular weight was decreasing indicating bulk degradation. Also the molecular

weight of amorphous polymers decreased faster than that of crystalline polymers. Molecular weight of bulk crystalline samples (C and P1) decreased faster compared to porous polymer samples, which can be due to autocatalysis. However, with amorphous polymers (P2 and P3) no significant difference was observed.

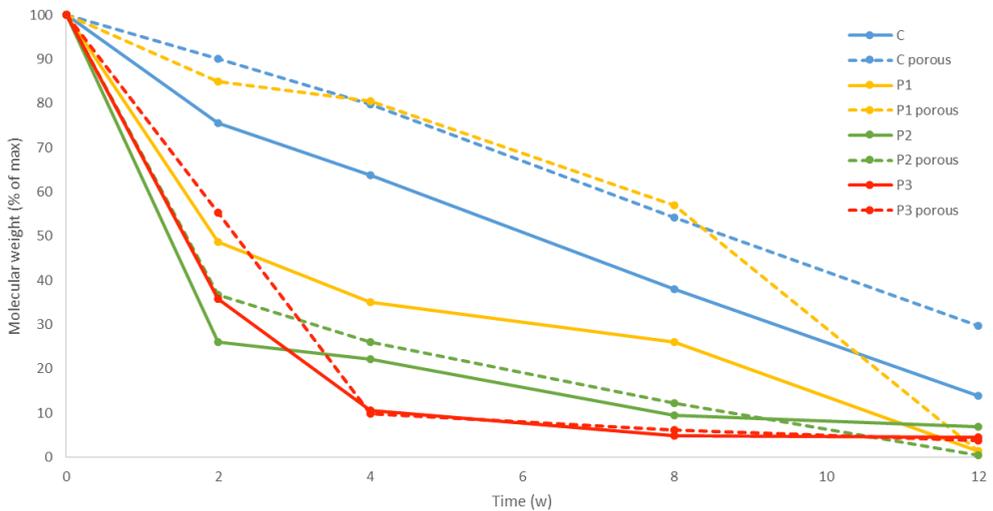


Figure 2: Number average molecular weight (% of maximum) of samples as function of hydrolysis.

CONCLUSIONS

The amorphous polymer samples degraded faster than partially crystalline polymers. The degradation behaviour of porous commercial polymer C and synthesized polymer P1 seem the most promising.

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ACELLULAR GRAFTS FOR ESOPHAGUS RECONSTRUCTION: AN ELECTROSPINNING TECHNIQUE APPROACH

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Keywords: nanofiber, electrospinning, esophagus, polylactide-co-polycaprolacton

INTRODUCTION

Regenerative approaches to solve esophageal defects or pathologies as gastroesophageal reflux and Barrett esophagus are in study [1]. The nanoscale fibers, obtained by electrospinning technique, present several interesting features such as high area/volume ratio and superior mechanical properties (stiffness and tensile strength), that can be exploited for esophagus regenerative purposes. The study is aimed to evaluate the feasibility to develop electrospun nanofiber matrices (mats) useful as acellular or cellular graft/flap for esophageal reconstruction.

Polyesters have been selected as the biodegradable and biocompatible polymers. Polylactide (PLA, MW: 234.454 Da) polycaprolacton (PCL, MW: 125.000 Da) polylactide-co-polycaprolacton (PLA-PCL 70:30, MW: 178.819 Da) have been processed by electrospinning apparatus NANON 01.

10-15-20-25 % w/w solution of PLA, PCL and PLA-PCL in CH_2Cl_2 /DMF were pumped through a syringe with a 18 and 27 gauge needles at 1 mL/h flow rate. The distance from the end of the needle to the collector plate was fixed at 15 cm. The voltage was imposed at 20,0 kV to obtain a stable Taylor cone. Increasing electrospinning times have been tested from 10 to 60 minutes in order to evaluated fiber behaviour and mechanical resistance at different thickness. An on purpose insulating cover tool

(ICT) was designed in order to achieve mats with defined geometry. The ICT was placed on the electrospinning plane plate collector.

The starting polymer solution viscosity was tested by Kinesus Rheometer (Malvern) and correlated to fiber entanglement and size. The mats have been characterized for their morphology (SEM), cytocompatibility (MTT test), 28 days *in vitro* degradation test in simulated physiologic conditions (PBS pH 7.4) and in pathologic conditions (PBS added with HCl 0.1M) at 37°C, by GPC monitoring polymer Mw. The mechanical properties were tested by an Enduratec ElectroForce® 3200 apparatus (Bose Corporation Eden Prairie, MN, USA).

RESULTS AND DISCUSSION

Results showed that polymer and solvent composition affect fiber formation both as size and shape. The best results in terms of regular fiber formation have been obtained with PLA 10%w/w in CH₂Cl₂/DMF 70:30 and PLA-PCL 70:30 25%w/w in CH₂Cl₂/DMF 70:30. Increasing polymer concentration and viscosity resulted in larger fibers up to 1 μm. *In vitro* degradation test results, in both the tested conditions, showed all the mats are stable for 28 days. These matrices showed higher cell attachment and proliferation with respect to scaffold obtained by solvent casting technique used as control. Elastic modulus values of electrospun mats were always compatible with esophageal physiologic pressure (3-5 kPa) [2].

SEM analysis showed suitable nanofiber obtained with PLA and PLA:PCL 70:30.

CONCLUSION

Electrospinning technique was set up in order to achieve fibers in the nanosize scale and reproducible polymer matrices. The insulating cover tool permitted to achieve mats with defined geometry, smaller fiber size and ordinate fabric, but it did not lead to reduce fiber density.

Both PLA and PLA-PCL 70:30 resulted in mats with suitable mechanical and biological properties.

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CITOTOXICITY OF SILICON/SILICA NANOWIRES ON THE BGM CELLS: A PRELIMINARY STUDY TO CARRY VIRUSES INSIDE NOT PERMISSIVE CELLS.

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BACKGROUND

Human noroviruses (HuNoVs) are a leading cause of acute, nonbacterial gastroenteritis worldwide. Young children and the elderly are at increased risk for more-severe and prolonged illness leading to hospitalization, while the disease is increasingly recognized as an important cause of chronic gastroenteritis for immunocompromised patients. NoVs are divided into six genogroups (GI–GVI). The GI and GII genogroups are the most important for human infection, HuNoVs are responsible for approximately 21–23 million gastroenteritis cases and 800 deaths in the USA and over 218,000 deaths in developing nations annually, mostly in children less of 5 years of age. Widespread vaccination that minimizes overall norovirus disease burden would benefit the entire population, but targeted vaccination of specific populations such as healthcare workers

may further mitigate the risk of severe disease and death in vulnerable populations. Despite the clinical importance of these viruses, relatively little is known about their pathogenic mechanisms. One of the most notable obstacles to investigating HuNoVs has historically been their uncultivability. The lack of a cell culture system and smaller animal model has delayed the development and commercial availability of vaccines and antiviral drugs.

OBJECTIVE

The aim of this preliminary study is to analyse the possibility of using the nanotubes of silicon/silica as nano-carriers to introduce Noroviruses inside the not-permissive cells. In particular, the final goals are to produce a new progenia of Norovirus to better understand the molecular biology of the virus; to prepare a possible vaccine; to produce more specific and sensible kits of diagnostic tools for environmental virology.

MATERIALS AND METHODS

It was used a 25 cm² flask of cells BGM buffalo green monkey cells (monkey kidney cells, a continuous line). The cells were detached and suspended with DMEM, and on each slide were distributed 3-4 ml of cells. The slide was placed on a Petri dish of the proper size and the dish was incubated at 37 C at 5% CO₂, left for 24 h. Then the slide was rinsed in saline solution and stained with hematoxylin-eosin solution.

HISTOLOGIC STAINING:

The cells were fixed with alcohol 80% for 5 min, and air dried. They were then stained with hematoxylin-eosin, 3 min of incubation, following washing in running water for 3 min. The cytoplasm is colored in pink with eosin for 30 sec, followed by washing in H₂O, dehydration with increasing alcohol 70, 95, 100, xylene (diaphanizing) and assembly with resins and application of cover slips.

SILICON/SILICA

Silicon nanowires have been grown on a commercial oxidized silicon wafer by using an ultra-thin gold seed layer of 2 nm as catalyst. The gold film was evaporated on the substrate and then the nanowires have been obtained in a plasma enhanced chemical

vapor deposition chamber at a temperature of 350°C and a working pressure of 1 Torr via a vapor-liquid-solid (VLS) technique starting from a gas mixture of silane and hydrogen. The silicon nanowires were naturally covered by a native layer of silicon oxide and the nanostructures were detached from the holder by using an ultrasonic bath for 5 min. The silicon nanostructures were diluted in water at a concentration of 130 mM.

EXPECTED RESULTS

- Step 0: The nanostructures have been conceived to be fabricated by using scalable techniques. In particular, the employment of the plasma enhanced chemical vapor deposition technique can ensure the manufacturing of the silicon nanowires on large area, thus reducing the material cost and providing a valuable tool that can be potentially industrialized in the biomedical field. The obtained nanostructures show the expected morphology and they can be tuned by changing some deposition parameters offering a wide range of material sizes according to the specific application.
- step 1: morphological evaluation by optic microscope of the cell monolayer on the distribution of the silicon/silica nanowires. It would seem that there is no cell toxicity, in fact the cells monolayer appears homogeneous with not damaged membrane.
- step 2: In the second phase of this preliminary study it will be possible to analyse how the silicon could go inside the cells. In this way it will be possible to evaluate the toxicity of the silicon, by using the fluorescence microscopy, thanks to the fluorescein binded to the silicon.
- step 3: If we have the proof that silicon goes inside the cells, it will be possible to substitute the fluorescein with the virus binded to the silicon. Silicon nanotubes will be destroyed in fragments by ultrasound, and these fragments will be added in the cell media to treat the cells

CONCLUSIONS:

Although other nanomaterial have been already reported (such as carbon nanotubes) as carrier for viruses, silicon/silica nanostructures can be easily manufactured, thus allowing a further step for device in biomedical field. These tools can be used for simplify the biological processes, especially in obtaining vaccines for human

pathologies. Indeed the idea of inserting in cells not cultivable viruses, such norovirus, adopting the nanostructures as valuable vector can permit the investigation of the virus metabolism.

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FABRICATION OF POLYPYRROLE NANO-FIBERS THROUGH INTRODUCING BIOINSPIRED DOPAMINE TO ACCELERATE HYDROXYAPATITE CRYSTALLIZATION

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Keywords: nano-fibers; polypyrrole; titanium; dopamine; osteointegration.

INTRODUCTION

Osteointegration at the interface between host tissue and implants plays an important role in the clinical success of dental and orthopedic implants such as those made from biomedical titanium. Inspired by the nature, simulating the performance and structure of bone is a promising strategy to improve osteointegration at interface.^[1] Moreover, electroactivity is a critical property for biomaterials because electronic systems can be integrated with the body. Conductive polymer (CP) with bone-like nanoarchitecture (i.e., nano-fibers) is an ideal candidate for modifying implants because they possess electroactivity, biocompatibility, biomolecule affinity and environmental stability.^[2] The presence of hydroxyl groups at the ortho-position of dopamine (DA) makes this group ideal for chelating many di- or tri-valent metal ions to form stable complexes.^[3] Therefore, DA facilitates the formation of calcium phosphate biominerals and can be applied to modify the biomaterial surface to improve osteointegration. Therefore, fabrication of polypyrrole nano-fibers through introducing bioinspired dopamine to improve hydroxyapatite deposition is universal a route to enhance osteointegration.

RESULTS AND DISCUSSION

By employing polydopamine (PDA) which could synthesized through electropolymerization in constructing the nano-fiber conducting polymer polypyrrole in mild medium, phosphate buffered saline (PBS), via template-free electrochemical polymerization, a high density of Ppy/PDA nano-fiber whose diameter were about 180nm were successfully synthesized (Figure 1a). The nano-fiber film, formed on pre-nucleation film (PNF) coating on the biomedical titanium to achieve a large specific surface area, maybe served as bread of extracellular matrix (ECM). To demonstrate our assumption that PPy/PDA nano-fibers formed with the assistance of PDA could facilitate the hydroxyapatite crystallization, PPy/PDA nano-fibers were applied for in vitro biomineralization in a simulated body fluid (SBF). Surface-anchored catecholamine moieties in PDA enrich the interface with calcium ions. This facilitates the formation of hydroxyapatite crystals that align with c-axes and are parallel to the PDA layer, as observed in natural hydroxyapatites in mineralized tissues. As shown in Figure 1a, abundant hydroxyapatite (HAP) was coated on PPy/PDA nano-fibers after 3 days of biomineralization in SBF. Moreover, the molar ratio of Ca to P was 1.62 (close to 1.67 in natural bone mineral) on PPy/PDA nano-grain. These results indicated that HAP nucleated and grew on PPy/PDA, which suggests it could be used as a bionic implant surface for increased osteointegration via improved osteoconductivity and even osteoinductivity.

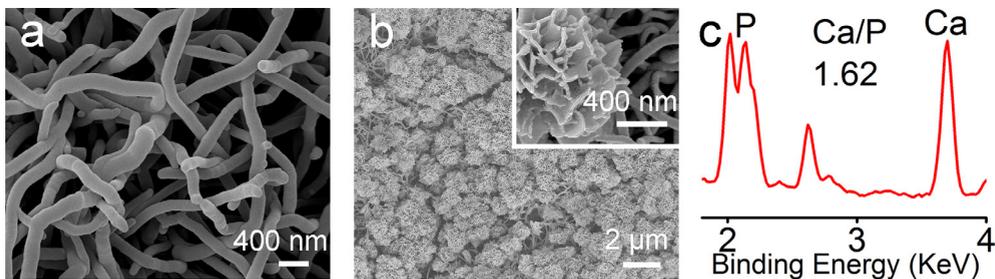


Figure 1: Field emission scanning electron microscopy (FE-SEM) images of PPy/PDA nano-fibers (a) and in vitro biomineralization in SBF for 3 days on PPy/PDA nano-grains (b) and the corresponding energy-dispersive X-ray (EDX) spectra (c) of in vitro biomineralization. Inset: (b) are high magnification images of randomly selected regions.

CONCLUSIONS

In summary, to construct a conductive polymer as an intelligent electrical implant surface and as bio-inspired adhesive proteins, DA, a small biomolecule found in brain, was used to develop a green fabrication approach for nano-fiber CPs on bone implants. PPy nano-fibers used for in vitro biomineralization in SBF demonstrated enhanced bioactivity. Surface-anchored catecholamine moieties of doping polydopamine enriches the conducting polypyrrole interface with calcium ions, accelerating the formation of hydroxyapatite crystals as observed in natural hydroxyapatites in mineralized tissues.

ACKNOWLEDGMENTS

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CULTURE PATCH METHOD FOR PRIMARY HIPPOCAMPAL NEURONS CULTURE

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Keywords: Nanofiber, Patch, Hippocampus neuron

INTRODUCTION

Most neuroscientists work with primary cultures, especially the widespread culture of hippocampal neurons, due to the relative simple nerve cell population as well as the expression of neural key phenotypic features [1]. However, the primary neuron culture relying on conventional planar substrates, such as culture dish and glass slides, does not provide neither the requested in vivo neural microenvironment nor the desired robustness and flexibility. S. Kaech et al [1] reported a “sandwich” culture of hippocampal neurons by plating them above an astrocyte feeder layer which was immobilized on glass slide. This approach is ingenious but the cells still attached on 2D and rigid substrate. Based on the results of our previous work on long term culture of human induced pluripotent stem cells (hiPSCs) on nanofibrous gelatin substrates [2] and hiPSC differentiation on crosslinked monolayer nanofibers [3], here we propose to culture primary hippocampus neurons on a monolayer net of gelatin nanofibers electrospun and crosslinked on a poly (ethylene glycol) diacrylate (PEGDA) honeycomb micro-frame.

RESULTS AND DISCUSSION

Figure 1 (A-D) shows the schematic process flow of culture patch fabrication. The PDMS mould for PEGDA frame (Thickness: 50 μ m; pitch size: 500 μ m; bandwidth: 50 μ m) was fabricated by soft lithography. Then a PEGDA solution mixed with 1v/v% Irgacure 2959 as photo-initiator was prepared and used to fill the PDMS mould-glass cavity by degassing induced micro-aspiration, followed by UV exposure. For easier handling, 100 μ m thick PEGDA rings prepared in a similar manner was mounted on the network, using pre-cured PEGDA solution as binder for UV curing. Prior electrospinning, the back face of PEGDA network was sputtered with 10nm thick Au to enhance the attachment of gelatin nanofiber and PEGDA network. 10 wt% gelatin solution was electrospun on the golden face of frame, followed with a crosslinking by 0.2 M EDC and 0.2M NHS for 4 h. Figure 1 (E) shows a photograph of a culture patch.

From the SEM images (Figure 2), after crosslinking, the gelatin nanofiber formed a monolayer net-like structure with pore sizes less than 8 μ m and a porosity of $62.27\pm 3.12\%$ measured with imagej software, upon which cells can be supported with minimum exogenous material contact and maximum exposure to the culture medium but enough supporting due to the pore size is smaller than cell size. Then we cultured brain sourced U87-MG cells on culture patch. From the SEM images (Figure 3), the bottom surface of cells was exposed through the spaces among highly porous nanofibers, which is totally different from conventional planar substrates. Interestingly, we also found the nuclei were mostly on the topside of nanofiber without migration to the bottom side of nanofiber. Previously, we reported the advantage of the culture patch for neuron differentiation [3]. Here to study the effect of culture patch for primary culture, hippocampal neurons were isolated from postnatal wistar rats (P1-P3) and seeded on laminin coated culture patch. From the immunofluorescence results (Figure 4) of cells stained with TUJ-1 and GFAP after 8-10 days in vitro (DIV), good integration and viability of neurons and glial cells can be observed. Glial cells present an in vivo like shape, which is mainly due to the in vivo extracellular matrix like gelatin nanofiber and the enhanced freedom for cell self-organization and neuron-glia interaction.

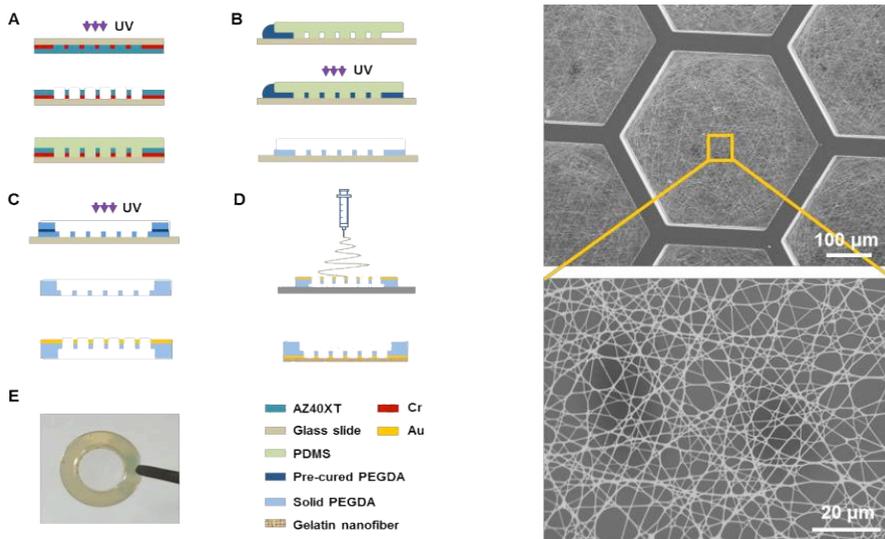


Figure 1: Culture-patch fabrication. (A) Mold fabrication by backside UV lithography and PDMS casting; (B) Fabrication of PEGDA honeycomb microframe by aspiration-assisted molding. (C) Binding of a PEGDA ring and backside Au deposition. (D) Electrospinning of gelatin nanofiber. (E) Photograph of a culture patch handled with a tweezer.

Figure 2: SEM photographs of the culture patch. After electrospinning and crosslinking, the nanofibers on PEGDA microframe formed a monolayer net with high porosity and pore size smaller than 8 μm .

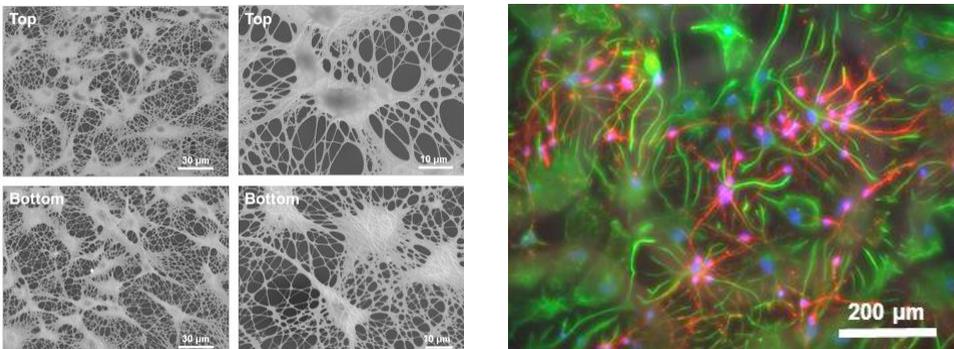


Figure 3: SEM images of U87 cells on culture patch after 48 h culture from top-view and bottom-view, respectively.

Figure 4: Immunofluorescence images of primary hippocampal neurons on the patch after seeding for 8-10 DIV, stained with GFAP (green) and TUJ1 (red).

CONCLUSIONS AND/OR OUTLOOK

In conclusion, our culture patch method is flexible and reliable for primary hippocampal culture and can be used as new substrate for *in vitro* studies in a three-dimensional environment.

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TAILORING OF MECHANICAL PROPERTIES BY MOLECULAR ORIENTATION IN POLYMERIC SCAFFOLDS

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Keywords: molecular orientation, scaffolds, fibres, tissue engineering

INTRODUCTION

Biomedical engineering is a multidisciplinary field which involves the 'application of the principles and methods of engineering and life sciences towards the fundamental understanding of structure-function relationships in normal and mammalian tissues in order to and the develop biological substitutes that restore, maintain or improve tissue functions' [1]. The mechanical properties of polymer implants show direct influence on cell activity and proliferation from the time of initial in vivo placement until final disposition [2]. The stretching of polymer molecules and structure organizing, has a major effect on the increase of the Young's modulus of the fibres [3]. Several of the dynamic function of cells is regulated by external factors coming from extracellular environment. In microscale, the surrounding microenvironment provides a medium in which cells move, grow, organize and differentiate to form tissues. The cell-material interactions are one of the most important issues in tissue engineering. In biomedical implants, mechanical forces transduced through the microenvironment alter the morphology and genetic expression (i.e. cytoskeleton) of the cells [4]. Additionally, mechanical properties of the whole scaffold decide about it application as suitable for tendon or ligament reconstruction.

The aim of this presentation is to study the possibilities of mechanical properties tailoring of polycarolactone scaffold by changing the molecular structure, especially molecular orientation

RESULTS AND DISCUSSION

Samples of polycaprolactone fibres (PCL, $M_w = 80,000$ g/mol, Sigma-Aldrich Co.) were formed by electrospinning technique. As a solvent 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Fluka Co.) was used. The fibres were collected on drum collector. The collector rotation speed was set as: 200, 550, 1000, 1300, 1700 rpm.

Morphology of the fibres was determined by scanning electron microscopy (SEM) using 2kV accelerating voltage at 10mA current flow. Molecular structure was determined by wide angle X-ray scattering(WAXS). Mechanical properties have been measured in uniaxial tensile test with a 100 N load and cross-head speed of 10 mm/min.

Morphology of electrospun fibres strongly depends on the rotation speed of the collector as it is shown on Figure 1. Higher rotation speed influence on PCL fibres ordering (parallel fibres) and decrease of its diameter. Random organised fibres are formed in case of low rotation speed of the collector (200rpm). The random organized fibres indicate inhomogeneous morphology and average diameter about 600nm. With the increase of rotation speed, fibres diameter decrease to about 300 nm This fact is related with a cold drawing process observed for fibres solidification using collector with higher rotation speed [5].

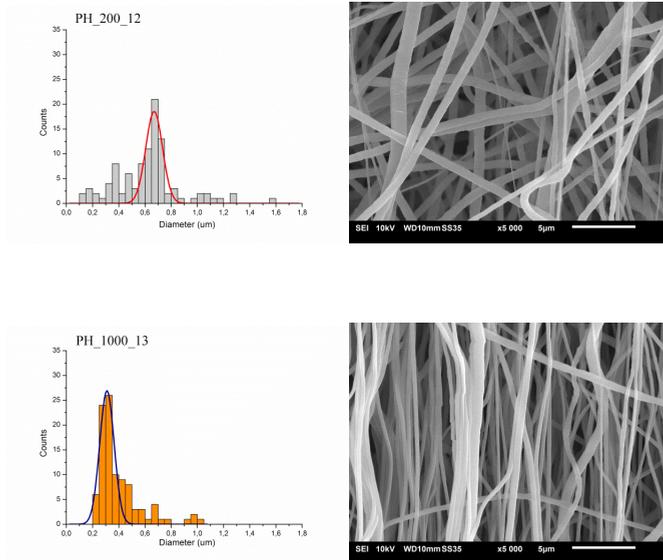


Figure 1: Morphology of electrospun fibres, collected on drum with a) 200rpm and b) 1000rpm

Molecular structure was characterised by molecular orientation from WAXS measurement. Fibres collected with low collector speed indicate low molecular orientation or no orientation. The Herman's orientation factor (f_c) for c axis of PCL crystals in fibres increase. It has been reported that chain orientation is significant in the early part of the straight jet section and it is trapped by drowning above 550rpm of the drum rotation.

Mechanical properties of the fibres are tailored by its morphology-fibres ordering. In case of nonordered fibres, elastic modulus and maximum tensile strength is few times lower than in case of ordered fibres (about three and six times respectively). Elongation at break of ordered fibres in comparison to nonordered decrease to half value.

CONCLUSIONS

Electrospun fibres prepared with different collector rotation speed, indicate different molecular orientation, influencing on its mechanical properties. We think that, beside common opinion, that surface effects are most significant for elastic modulus, supramolecular microstructure have crucial meaning for deformation behaviour of the nonwoven during strain-stress process. It is in agreement with the concept of Arinstein et.al, who state that the scale of supramolecular structures in polymer fibres results in noticeable variations in their mechanical and thermal properties [4].

In general PCL electrospun nanofibers indicate relatively low molecular orientation, resulting from competition between the extensional forces and orientation relaxation [5]. However we proved that even small changes of molecular orientation strongly influence on mechanical properties of PCL nonwovens.

ACKNOWLEDGEMENTS

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BIOCOMPATIBLE HIERARCHICAL POROUS SCAFFOLDS OF MAGNESIUM SILICATE/ POLY(BUTYLENE SUCCINATE) COMPOSITE FOR BONE REGENERATION

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Keywords: Scaffolds, mesoporous materials, composites, tissue engineering, bone regeneration

INTRODUCTION

The addition of magnesium (Mg) in ceramics or glasses can modify their physical, thermal, and mechanical properties ^[1]. Additionally, Mg plays a significant role in new bone formation ^[2]. Mg-containing silicate materials, including bioceramics and bioactive glasses (BGs), are therefore attracting increasing interest for biomedical applications. Mesoporous materials, exhibiting well-organized porous structures, tailorable pore size, large specific surface area and high pore volume, are fascinating materials for a large number of applications including drug delivery ^[3]. Mg-containing mesoporous BGs, therefore, are considered to be promising materials for either bone regeneration/repair or drug delivery ^[1,4].

Biodegradable polymeric materials have been used to assemble 3D porous scaffolds for bone tissue engineering. Poly(butylene succinate) (PBSu), one of biodegradable aliphatic polyesters, is a suitable material for scaffolds, due to their excellent biocompatibility, good processing ability, as well as their non-toxic degradation by-products ^[5]. However, PBSu is hydrophobic that affects cell attachment and infiltration. Furthermore, PBSu exhibit slow degradation rates and lack apatite-forming ability ^[6].

Based on the above rationale, we hypothesized that the incorporation of mesoporous magnesium silicate glasses (m-MS) into PBSu scaffolds could overcome the above limitations of using PBSu alone as bone scaffolds. In this study, we synthesized m-MS/PBSu composite scaffolds with hierarchical macro-micro-mesoporous structure using a simple solvent-casting and salt leaching method. The effects of the incorporation of m-MS on pore structure, hydrophilicity, bioactivity and *in vitro* degradation were investigated. Moreover, in order to evaluate the *in vitro* bone regeneration potential, the cytotoxicity, cell attachment, and osteogenesis activity of scaffolds were evaluated.

RESULTS AND DISCUSSION

Nitrogen adsorption-desorption isotherm of m-MS, exhibiting Type IV adsorption behaviour with a clear H1-type hysteresis loop according to the IUPAC classification, proves the mesoporous nature of m-MS. Their BET specific surface area and pore volume were 551 m²/g and 0.62 m³/g, respectively. Additionally, m-MS exhibited a pore size of approximately 8 nm that was determined by BJH measurements. The mesoporous structures of m-MS were further confirmed by high-resolution transmission electron microscope (HRTEM).

The m-MS/PBSu composite scaffolds containing 20 and 40 w% m-MS were prepared and designated as C20 and C40. Pure PBSu scaffolds were prepared as control (C0). XRD patterns and FTIR spectra of C20 and C40 scaffolds confirmed the successful incorporation of m-MS in PBSu. The images of synchrotron radiation-based mCT (SRmCT) show that all scaffolds exhibited a macroporous structure without obvious visual difference. More importantly, the composite scaffolds exhibited interconnected macropores and a highly uniform pore distribution.

SEM images of the scaffolds show that the pore size of all the scaffolds was approximately 400 μm, which was consistent with the size of salt particles used to generate pores. The results also indicated that no obvious difference in pore size was observed, but the surface of scaffolds became relatively rough after the introduction of m-MS. The water absorption ability of scaffolds increased with the increase of the added content of m-MS, while the compressive strength of scaffolds increased from 2.2 MPa to 4.1 MPa with the m-MS content increasing from 0 to 40 wt%. Needle-like apatite particles could be found on the surfaces of C20 and C40 scaffolds after immersion in simulated body fluid for 5 days, while no deposition could be found on C0 scaffolds.

The viability of MC3T3-E1 cells was evaluated by the CCK-8 assay, showing that

the viability of C20 and C40 groups were significantly higher than that of C0 group. CLSM images of stained MC3T3-E1 cells showed that no obvious dead cells could be observed on C40 scaffolds and living cells dispersed homogeneously on C40. CLSM images of MC3T3-E1 cells stained by FITC and DAPI also showed that the formed actin cytoskeleton distributed irregularly on C0 scaffold, whereas the cells spreading on C20 and C40 scaffolds exhibited a typical fibroblastic morphology with more cytoplasmic extensions and filopodial attachments. The relative proliferation rates of cells on all scaffolds were increased over culture time. At day 3, the relative proliferation rate of cells on C0 was significantly lower than that on C40, while no obvious difference was found between C40 and C20 groups. ALP activity of MC3T3-E1 cells on scaffolds showed that the ALP activity of cells on C40 was significantly higher than those of C20 and C0 scaffolds at day 7 and 10. The ALP activity of cells on both C40 and C20 were obviously higher than that of C0 at day 14.

CONCLUSIONS

In this study, biodegradable m-MS/PBSu scaffolds with well-defined hierarchically macro-micro-mesoporous structure were successfully fabricated for bone regeneration. The incorporation of m-MS into PBSu could enhance the compressive strength, hydrophilicity, and bioactivity of scaffolds. Higher content of m-MS in the scaffolds could lead to increased cell viability and proliferation. Cell attachment and distribution were also improved on the m-MS incorporated PBSu scaffolds in comparison to non-modified PBSu scaffolds. In addition, the ALP activity of cells on C40 scaffolds was evidently the highest compared to the C20 and C0 scaffolds, demonstrating the osteogenic potential of C40 scaffolds. More detailed cell biological experiments will be performed in future to evaluate the in vitro and in vivo bone regeneration potential of m-MS/PBSu scaffolds.

ACKNOWLEDGEMENT

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ION BEAM EFFECTS ON CHITOSAN MEMBRANES FOR TISSUE ENGINEERING APPLICATIONS

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Keywords: Chitosan, Protein adsorption, biodegradation, ion implantation

INTRODUCTION

Chitosan has been studied for various practical applications because of functions such as biodegradability, low toxicity, and acceleration of fibroblast formation in animal body, acceleration blood clotting, drug delivery, antimicrobial activity and high solubility in water. In this study, we attempted to prepare and characterize the chitosan membrane surfaces induced by metal-gas (MEVVA) ion implantation.

RESULTS AND DISCUSSION

Chitosan membranes were prepared in two different microstructures to investigate the structure effects on the protein adsorption and in vitro degradation. Dense and asymmetric chitosan membranes prepared by dissolving in acetic acid solution. For dense membrane production, solvent casting method was used. For asymmetric membrane preparation dry/wet phase separation method was used by using 20 minutes pre treatment time. By changing this time pore size and thickness of the membrane is changed that also effects the membrane properties like diffusion ratio, water absorption, degradation time etc. Chitosan membranes then were implanted by C and C+N ions by using MEVVA ion implanter. As a result of these, we investigated the effect of ion implantation on the protein adsorption behavior, in vitro degradation

and cell attachment properties of chitosan films before and after the ion implantation. The chitosan films were prepared by solvent casting method for dense films, and dry/wet phase separation method is used to obtain asymmetric chitosan membranes. Characterization studies of these membranes were performed by using Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and Differential Scanning Calorimetry (DSC).

CONCLUSIONS

The ion implantation effect on ion beam modified chitosan membranes for neural cell attachment (B35) is also examined for different dose and energy parameters of the beam. As a result of that, surface modification by ion implantation with 1016 cm^{-2} dose, and 1 pps frequency, 20 kV acceleration voltage were the most appropriate values in order to neural cell attachment and neurite extension capability with chitosan membranes.

ANTI-QUORUM SENSING ACTIVITY OF KAEMPFEROL LOADED LECITHIN/CHITOSAN NANOPARTICLES

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Keywords: Flavonoids, Encapsulation, Nanoparticles, Anti-Quorum Sensing, Sustainable Nanoagents .

INTRODUCTION

Many bacterial species communicate with each other in a population-dependent mechanism to regulate their physiological functions. This mechanism is called as quorum sensing (QS) which is mediated through the release of diffusible and small signaling molecules called as autoinducers [1]. Quorum sensing (QS) mechanism plays a vital role in many bacterial species, which is found to be implicated in various factors including bacterial pathogenicity and biofilm formation. Therefore interrupting the QS mechanism may be an attractive strategy to develop novel QS-based anti-bacterial drugs [2]. We investigated the quorum sensing inhibitory activity of kaempferol loaded lecithin/chitosan nanoparticles (KAE-LC NP) to compare pure kaempferol in the case of the time dependent stability. The KAE-LC NPs were prepared by using the electrostatic self-assembly technique and characterized in terms of average size, surface charge, morphology and chemical structures. Moreover, in vitro evaluation of the KAE-LC NP system was determined by the anti-quorum sensing activity

against *Chromobacterium violaceum* CV026 in a concentration- and time-dependent manner compare to free KAE.

RESULTS AND DISCUSSION

KAE-LC NPs have diameters of 270 ± 10 nm, PDI ≤ 0.2 and net positive surface charge ($+56 \pm 4$ mV) respectively, and are spherical in shape and uniform. It was observed from FTIR spectrum that encapsulation of kaempferol in the KAE-LC NPs was formed by hydrophobic interactions. The KAE-LC nanoparticles at all tested concentration inhibited the production of violacein pigment in *C. violaceum* CV026. In addition, encapsulated KAE in nanoparticle exhibited a significantly anti-quorum sensing inhibition efficacy (75%) against *C. violaceum* at the end of the 45 day storage period. However, free KAE displayed no inhibitory activity at the end of 15 day storage period.

CONCLUSIONS AND/OR OUTLOOK

The obtained results exhibited that the formulated encapsulated kaempferol into chitosan nanoparticles at different storage time periods effectively regulated bacterial phenotypes like violacein pigmentation and biofilm formation when compared with pure kaempferol. These findings lay a foundation for the utilization of the KAE-LC nanoparticles as QS-based sustainable anti-biofilm agents to manage bacterial communication inhibition.

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STABILIZATION OF LEMONGRASS OIL NANOEMULSIONS BY AN IONIC AMPHIPHILIC DERIVATIVE OF CHITOSAN

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Keywords: nanoemulsions, essential oil, ionic amphiphilic chitosan

INTRODUCTION

N-acyl modified chitosans obtained by covalent conjugation of hydrophobic moieties and amino groups of the polysaccharide are largely described in the literature [1].

Another approach, recently proposed, involves the preparation of hydrophobically modified chitosan (HM-Cs) by electrostatic interaction between the amino groups of chitosan, and fatty acids, in particular oleic acid. The resulting chitosan derivative gives polymeric micelles suitable to improve the solubility of hydrophobic poorly soluble drugs [2, 3].

The use of this derivative has been proposed to stabilize emulsions of lemongrass essential oils (LG) in water [4]. In the present work the nanoemulsions based on HM-Cs were studied in terms of drug loading and physical stability by assessing dimensions at definite times.

RESULTS AND DISCUSSION

Emulsions were prepared by adding oleic acid and lemongrass essential oil in two oil:HM-Cs weight ratios (0.5:1 and 1:1) to chitosan HCl solutions at different concentrations (from 0.05 % to 0.5% w/v).

Drug loading, calculated as percentage from the ratio between amount of oil quantified (by UV measurements) in the emulsion and amount of HM-Cs used in the preparation, ranged between 5% and 30%, depending on the system, as illustrated in Figure 1. The nanoemulsions prepared with lower chitosan concentrations

(0.05% and 0.1% w/v) showed smallest particle size and highest LG loading. The final concentration of LG encapsulated was however lower, about 0.5 mg/ml, than in the case of emulsions prepared starting from high chitosan concentrations, where oil concentration resulted about 1.5 mg/ml. These levels could be further increased by sample concentration in a second step, although they are compatible with the levels used in many essential oil applications.

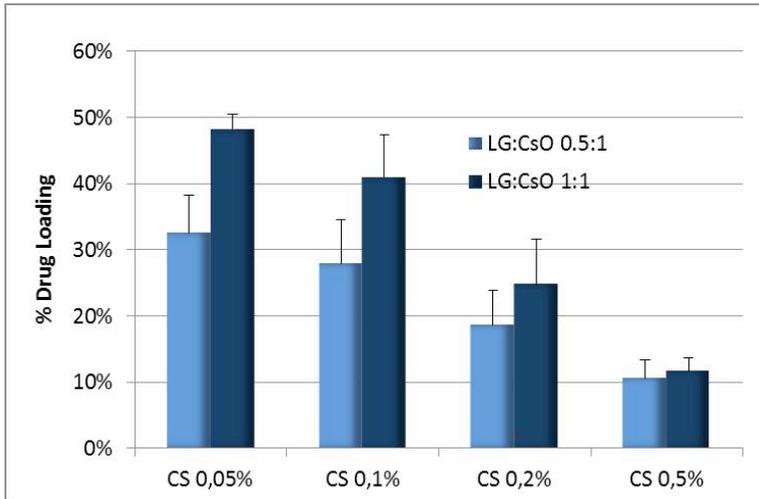


Figure 1: Drug loading (%) in the nanoemulsions

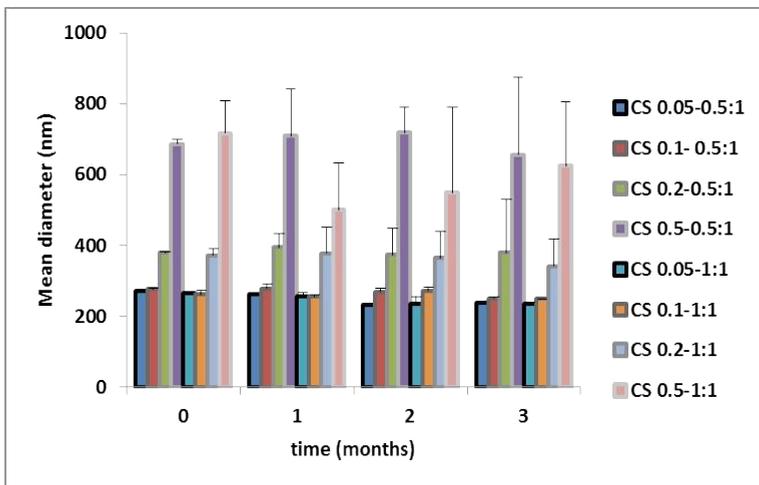


Figure 2: Dimensions of the nanoemulsions up to 3 months

The physical stability of the droplets in the preliminary test performed maintaining the samples at room temperature for up to three months showed no significant variations, as shown in Figure 2.

The occurrence of chitosan coating around the single droplets allowed moreover the emulsion to maintain the properties that are well described in the literature for the cationic polymer, such as for example mucoadhesive behaviour.

CONCLUSIONS AND/OR OUTLOOK

The method proposed to obtain the dispersion of LG oil in water and the HM-Cs derivative employed to stabilize them resulted in emulsions in the nanometers range with essential oil loading comparable with the levels found for different systems in the literature. The shell that chitosan forms outside the droplets gives the nanoemulsion the positive ability of interaction with the epithelia, especially at mucosal sites, that characterize the polysaccharide.

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BIOMIMETIC SURFACE FOR CONTROLLED CELLULAR RESPONSE

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Keywords: Lactoferrin, hydroxyapatite, inflammation, macrophages, biomimetic

INTRODUCTION

Bone fracture healing involves inflammation resolving. Macrophages are the main cells recruited at the site of injury involved in the modulation of the inflammatory processes [1]. Prolonged release of a key pro-inflammatory cytokine TNF- α by activated macrophages is associated with tissue damage and reduces bone repair and regeneration. Beside the mechanical properties, biomaterial scaffold must create an appropriate microenvironment that promotes specific cell growth and normal wound healing. By adding on biomaterial surface molecules known to have immunomodulatory properties [2], the cytokine balance response for favourable repair mechanism could be controlled. We used Matrix Assisted Pulsed Laser Evaporation (MAPLE) method to obtain hybrid coatings of natural compounds, lactoferrin (Lf), a known mediator of host response [3], and hydroxyapatite (HA), an inorganic component of bone. The aim of this study was to examine the cytotoxicity and inflammatory potential of HA-Lf biomimetic biomaterial, induced in an *in vitro* inflammatory model.

RESULTS AND DISCUSSION

Physical-chemical aspects of engineered biomaterial, morphology, topography, roughness and functional integrity of surface were assessed using AFM (atomic force microscopy), SEM (scanning electron microscopy) and FTIR (fourier transformed

infrared microscopy) methods which demonstrated successful uniform deposition of hybrid coatings and the maintaining of the structural integrity of functional groups in the MAPLE-deposited coatings.

Using an *in vitro* model of inflammation based on stimulation of the human leukemic monocytic THP-1 cells - differentiated to macrophages - with bacterial endotoxin, the potential inflammatory response of biomimetic coated biomaterial was assessed. Metabolic activity and cell proliferation increased in the case of THP-1 cells cultured on Lf-HA covered biomaterials compared to biomaterials covered with either Lf or HA, as shown by colorimetric MTS assay. The presence of Lf-HA combination on the surface of biomaterial attenuated the proinflammatory cytokine TNF- α release from macrophages attached on biomaterial surface after 18 hours stimulation with bacterial endotoxin. No cytokine release was observed from macrophages cultured on any of the surfaces in the absence of inflammatory stimulation as determined by an ELISA-typed method. Fluorescent actin imaging revealed that THP-1 differentiated cells attached and adhered on all surfaces exhibiting a mix round-shaped and spread morphology.

CONCLUSIONS

Our results indicate that physical and biological properties of Lf-HA coated materials influence macrophage behaviour and modulate inflammatory response. Smart design of biomaterial by combining biological components with low inflammatory response and excellent biocompatibility might be a suitable choice for therapeutic approach in biomedical bone applications.

ACKNOWLEDGMENTS

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IN VITRO STUDIES OF SHORT- AND LONG-TERM RESPONSE OF MC3T3-E1 PRE-OSTEOBLASTS TO GRAPHENE/SERICIN COATED SUBSTRATES

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Keywords: graphene, sericin, osteoblasts, matrix mineralization

INTRODUCTION

Conventional orthopaedic metallic implants suffer from a restricted lifetime which is partially caused by a poor osseointegration. Coatings represent a powerful mean of improving implant biological performance. Recently, graphene has been showed to provide pro-osteodifferentiation capability on implants and scaffold materials by surface modification [1]. Another suitable material for biomedical applications is sericin, which has been shown to enhance proliferation and attachment of mammalian cells [2]. In this context, the objective of this study was to investigate *in vitro* cell behavior of MC3T3-E1 pre-osteoblasts on composite graphene/sericin coatings obtained by Matrix Assisted Pulsed Laser Evaporation (MAPLE) technique, by studying cytotoxic potential of these coatings, cell adhesion, morphology, proliferation and differentiation potential.

RESULTS AND DISCUSSION

Uniform coatings, with variable roughness depending mainly on the target composition were obtained and characterized by different techniques (Fourier

transform infrared spectroscopy, Scanning Electron Microscopy, Atomic Force Microscopy). The structural characteristics of the coatings given by FTIR and the morphological characteristics (homogeneity, density and the roughness of the coatings) demonstrated the use of MAPLE as viable technique for obtaining sericin-graphene composite coatings for biological assays.

Labeling of actin and vinculin was used to evaluate the cell attachment and cytoskeleton organization in pre-osteoblasts grown on the analyzed substrates. The results indicated similar adhesion patterns of osteoblasts on both samples. Further, no differences in cell morphology and number were observed and there was no evidence of membrane damage, cytoplasmic vacuolation or cell death.

Figure 1: Pre-osteoblast differentiation on composite graphene/sericin coating after 2 and 4 weeks of culture as assessed by Alizarin red staining.

LDH release in culture medium was found to be low, suggesting that graphene/sericin coated substrates did not exert cytotoxic effects. Next, cellular survival and proliferation ability of MC3T3-E1 cells seeded onto graphene/sericin coatings were monitored at 2 and 6 days post-seeding by combining a qualitative method (cells staining with calcein AM and EthD-1) with a quantitative one (MTT colorimetric assay). A high percent of MC3T3-E1 cells stained with calcein AM and very few cells stained with ethidium bromide were found on the samples, indicating that these coatings sustained cell survival. Moreover, MTT assay showed that the cell number increased with cell incubation time, suggesting that analyzed coating promoted cell proliferation.

A hallmark of osteoblast differentiation both in vivo and in vitro is the formation of an extracellular matrix whose main component is calcium. Alizarin staining indicated that the composite coating was significantly more efficient in supporting mineralized matrix deposition upon osteogenic induction than the reference material.

CONCLUSIONS

This in vitro research indicates that the new graphene/sericin coating leads to improved biocompatibility. As we have shown, the coated material sustained cell adhesion, proliferation and stimulated extracellular matrix mineralization. In the context of biomaterials development for current bone implantology, these findings open new avenues towards the potential use of these innovative coatings for tissue regeneration purposes.

ACKNOWLEDGMENTS

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THE EFFECTS OF VERTICALLY, INTERCONNECTED CARBON NANOWALLS ON BACTERIA AND OSTEOBLASTS

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Keywords: carbon nanowalls, bacteria, osteoblasts, adhesion, morphology

INTRODUCTION

Titanium is first choice biomaterial for orthopaedic applications, due to its favourable mechanical properties, corrosion resistance, and biocompatibility. However, metallic implants have a limited ability to induce bone integration. Moreover, titanium implants are susceptible to infections. Recent studies show that an increasing number of implant revision surgeries are necessary each year, mostly due to implant related infection and poor osseointegration. This problem can be tackled by smart surface design. Thus, surface topographies which can be tailored to selectively discriminate between microbial and cell adhesion, potentially supporting the desired biological activity over long periods of time, are highly desirable for implant technologies. In this regard, the present study was designed to explore the potential of carbon nanowalls deposited on titanium by low pressure plasma to prevent microbial colonization while supporting the growth and proliferation of osteoblast-like cells.

RESULTS AND DISCUSSION

Scanning electron microscope analysis revealed the highly porous architecture of vertically oriented, interconnected carbon nanowalls. Considering this specific morphology, we hypothesize that carbon nanowalls surface may allow the

adsorption of higher levels of specific proteins (e.g. bone morphogenic proteins) that can potentially confer osteoinductive properties to the material. Further, carbon nanowalls were found to sustain the adhesion of *Pseudomonas aeruginosa*, but membrane damage in carbon nanowalls exposed bacteria were identified by morphological changes in the cell structure. In Figure 1 it can be seen that on carbon nanowalls substrate bacterial membrane show an abnormal texture like membrane rupture. Also, membrane blebs have been observed. Membrane damage may be caused by the sharp edges of carbon nanowalls, which could penetrate the cell membrane and physically disrupt its integrity.

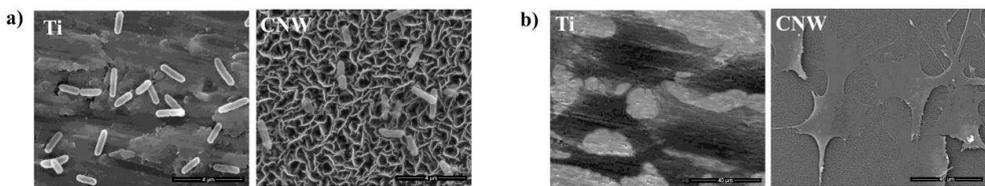


Figure 1: Scanning electron micrographs of a) *Pseudomonas aeruginosa* after 2 h incubation on titanium and carbon nanowalls coated titanium samples, and b) osteoblast cells 24 h post-seeding.

Initial cell attachment of osteoblasts was affected by surface topography, yielding more elongated cell morphology compared to a more spread, polygonal morphology displayed by cells cultured on titanium. Moreover, differences in cell morphology were accompanied by differences in cytoskeleton organization. One possible explanation for this behavior consists in the constraints on cell morphology due to nano-micro surface topography. Moreover, differences in surface chemistry and surface wettability between deposited carbon nanowalls and titanium would be anticipated to support differences in conformation of the proteins adsorbed to their surface that may also be important in influencing osteoblast morphology and function. Next, LiveDead assay revealed highly viable osteoblast cells on carbon nanowalls. Further, evidence of cell division provided by MTT assay confirmed uncompromised cell viability. Thus, although cell cytoskeleton was less organised and more diffuse on carbon nanowalls, cell viability and proliferation were not affected. Our results are in line with previous papers which demonstrated that graphene-based nanomaterials possess anti-bacterial activity, while preserving cell viability [1, 2].

CONCLUSIONS

Carbon nanowalls samples displayed a biocompatible surface in terms of osteoblast growth. At the same time, attachment of bacterial cells to carbon nanowalls – coated titanium samples was shown to induce a disruption of the cell integrity, reducing bacterial development on the surface. Overall, this study demonstrates potential applications of carbon nanowalls as coating material for controlling microbial and cell adhesion and development.

ACKNOWLEDGEMENTS

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CYTOKINE CONTENT OF HUMAN LYOPHILIZED AMNIOTIC MEMBRANE EXTRACT

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Keywords: amniotic membrane. Lyophilization, growth factors

INTRODUCTION

The fetal side of the placenta is covered by a thin membrane referred to as the amniotic membrane (AM). This membrane is increasingly being used as a biomaterial for several surgical processes due to its wound-healing properties with numerous studies confirming its effectiveness. The wound-healing properties have mainly been linked to the morphological characteristics as well as the cytokine and growth factor contents of amniotic membranes. It is therefore highly necessary that the ideal lyophilization methods are chosen in order to preserve if not improve the cytokine and growth factor composition of the amniotic membrane extracts and also prolong its storage. The absence of a standardized method means that different lyophilization methods result in lyophilisates with varying cytokine and growth factor content. This study there aims to carry out comparative analyses of two amniotic membrane treatment methods with respect to cytokine content of the resultant lyophilisates. The first method involved low frequency ultrasound treatment of amniotic membrane while the second method entailed liquid nitrogen treatment of AM followed by immediate boiling. Enzyme-linked immunosorbent assay (ELISA) was then used for measurement of growth factor concentrations in the obtained amniotic membrane lyophilized extracts.

RESULTS AND DISCUSSION

Analyses of 50 amniotic membrane samples showed no significant difference in the level of dryness of lyophilisates after treatment by both methods. The water content in the lyophilized AM extracts constituted less than 0.5%. Approximately 98% dry lyophilisates with fairly high concentrations of growth factors were obtained by both methods. A combination of both methods was seen to decrease EGF, PDGF-BB and TGF-beta-1 concentrations in the extracts. Ultrasound treatment of AM prior to dry-freezing resulted increased concentrations of the growth factors examined. FGF and TNF-alpha levels were approximately equal in both methods.

CONCLUSIONS

In conclusion the present results demonstrate to a certain degree that cryo-treatment of AM with a subsequent boiling step causes a greater protein loss compared to ultrasound processing. Ultrasound processing prior to freeze-drying can therefore be considered a better processing and preservation method for human amniotic membrane.

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ACTIVATED PLATELET-RICH PLASMA: CYTOKINE CHARACTERIZATION AND PROFILING

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Keywords: platelet-rich plasma, activation, growth factor, cytokine, preparation

INTRODUCTION

The use of platelet-rich plasma (PRP) currently spans across various science fields related to regenerative medicine. The effectiveness of PRP therapy is thought to be associated with increased concentrations of bioactive cytokines and growth factors that play a fundamental role in hemostasis and tissue repair. Granules present in platelets are reported to release secretory proteins such as FGF, TGF- β , TNF-alpha, EGF and PDGF after platelet activation, thus triggering tissue repair and regeneration through mechanisms vaguely understood [3]. Platelet activation concepts are reported to involve single spinning (SS) or double spinning (DS) of blood plasma with a subsequent low dose thrombin or calcium treatment. These variations tend to affect PRP quality in terms of cytokine content and biological activity [2]. Even though PRP therapy holds a lot of promise in tissue healing and regeneration and has minimal associated risks, the lack of a standardized protocol for PRP preparation poses methodological challenges to researchers. There is also very little known about the biomolecular nature and proportions of cytokines released by the various methods of platelet activation. We therefore, sought to optimize protocols for obtaining PRP by modifying the already existing Ariki et al method [1] and also characterize the cytokine content.

RESULTS AND DISCUSSION

Autologous platelet-rich plasma was obtained from 10 donors by the double spin method. This method involved an initial centrifugation of whole blood, collection of the upper plasma layer and a second centrifugation of this fragment. The precipitate was then re-suspended in appropriate volumes of Phosphate buffered saline (PBS) or platelet-poor plasma (PPP) prior to calcium activation. FGF, EGF, PDGF-BB, TNF-alpha and TGF-beta-1 levels in PRP were determined using ELISA kits. PRP preparation with PBS was shown to result in a more efficient secretion of growth factors than the PPP method. The double spin PRP preparation method was also seen to have an effect on cytokine release with an increase in FGF, EGF, TNF-alpha and TGF-beta concentrations by 3, 1.4, 5.7 and 2.2 folds respectively recorded.

CONCLUSIONS

Based on the obtained results, we concluded that different PRP preparation and activation protocols have varying effects the release of cytokine. A higher platelet concentration is obtained by the double spin method compared to the single spin method.

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BIOMIMETIC GIANT VESICLES ELECTROFORMATION

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Keywords: Membrane, Giant Unilamellar Vesicles, Electroformation, Liposomes,

INTRODUCTION

Giant Unilamellar Vesicles (GUV) are liposomes of 10 – 100 μm of diameter formed by a single phospholipid bilayer enclosing an aqueous core. Their cell-like size motivates the wide use of GUV as model membrane system, with the advantage to be visible under light microscope[1]. Among the existing methods of preparation, the most commonly used is the so called “electroformation” or “electroswelling” which consist in an hydration of a dried lipid film in presence of an alternating current electric field, which leads to an homogeneous population of spherical vesicles characterized by a narrow size distribution[2].

Despite their relevance as biomimetic systems, the underlying mechanism of GUV formation is still poorly understood and an univocal model explaining the vesicles formation is still lacking.

In this work, by using phase contrast microscopy has been performed a real time study on the kinetics of GUV growth. At the same time, has been characterized the structural and topological properties of electroformed vesicles through fluorescence and confocal microscopy, with the final aim to define a simple and intuitive model which connects the vesicles features with the electric field parameters, namely frequency, voltage and waveform.

RESULTS AND DISCUSSION

DOPC lipid vesicles were observed during the swelling, at varying frequency and voltages, through an inverted phase contrast microscope. The acquired images were analyzed through the *ZEN lite 2011* microscope software and the size as a function of time was collected.

As can be seen from Figure 1, the data shows a correlation between the vesicle's size and the field parameters. In particular, if the voltage is raised the size increases, while if the frequency is raised the size in time decreases.

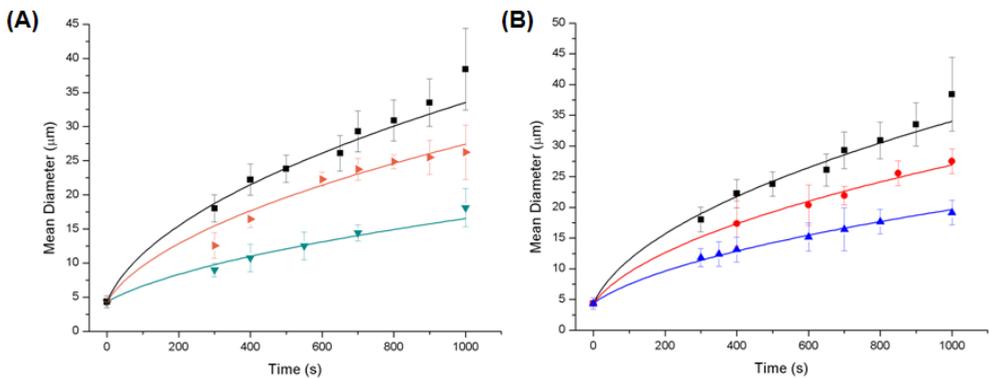


Figure 1: Mean diameter of the vesicles as a function of time. Panel (A) the curves refers to data acquired at a fixed frequency of 10 Hz and at different voltages: 5 V (black), 3 V (orange), 1 V (green). Panel (B) the curves refers to data acquired at a fixed voltage of 5 V and at different frequencies: 10 Hz (black), 30 Hz (red), 50 Hz (blue). The solid lines indicates the best fits

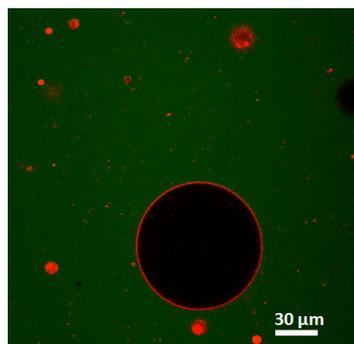


Figure 2: Confocal laser image of a GUV surrounded by calcein. The vesicle's membrane is impermeable to the external fluid.

These observations are successfully explained by an equivalent electric circuit model in which an equation governing the size growing in a “low perturbative” regime has been defined. Moreover from the analysis of the dynamics a growth rate, which depends both on the microscopic properties of the lipid molecules and on the electric field parameters, has been identified. For the same kind of lipid system the growth rate depends on the impulse transferred by the electric field to the lipid molecules.

CONCLUSIONS AND/OR OUTLOOK

The study of the electroformation process allows to characterize the phenomenon and to define the optimal experimental condition to produce spherical vesicles with an impermeable membrane and formed by a single phospholipid bilayer. The identification of a parameter, e.g. the growth rate, which specify the dynamical processes enable us to modify the formation condition based on the kind of lipid molecules. This is particularly relevant in the case of GUV formation starting from lipid mixtures or solution containing lipids and membrane peptides which affects the membrane fluidity.

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SILICON IMPLANTS PERMEABILITY'S TO ORGANIC FATTY ACIDS

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Keywords: breast implants; siloxanes; permeability; fatty acids; in vivo

INTRODUCTION:

Breast reconstructions with skin expander may diminish local complications (Spear and Spittler, 2001). There are no recommendations about an implanted time limitation for these medical devices. The main breast implant's aging factor is the lipids penetration into implants. The results of Adams *et al.*, focused on the link between triglyceride and phospholipids penetration with breast implant ruptures (Adams *et al.*, 1998). We investigated both the lipids penetration and the changes in silicone shells' mechanical resistance on explanted expanders after *in vivo* natural aging in women.

RESULTS AND DISCUSSION:

Twenty-eight breast expanders, from two different manufacturers, have been explanted and analysed. Data are sum up in table I.

Globally there is no correlation between maximum shell elongation and the implanted time, but, the sub-group analyses proved a difference between manufacturers. Allergan® shells' expander elongation decreases with time whereas Mentors' does not. The difference becomes significant after 1 year of implanted time.

Moreover, all expanders' saline serums contain fatty acids, cholesterol and other chemical compounds. Those chemicals are siloxane impurities and polymer or biological elements.

The fatty acid penetration through shells' expander begins immediately after the implantation. The shells' permeability is qualitatively the same for all expanders. Nevertheless, the exact kinetics of the penetration is not yet documented.

CONCLUSION AND OUTCOMES:

This scientific study brings new information about silicone shell aging *in vivo* for women with breast reconstruction surgery. First, the lipid aging presented by Adam *et al.*, seems to be more dependant of fatty acid whose can penetrate through the shell in a short laps of time. Secondly, the fatty acid and cholesterol penetration is not associated with a change of shell mechanic properties for all expanders. Finally, shells' expanders release some siloxane impurities in the serum inside the implant.

The siloxane impurities' mobility in the tissues near the implants have to be explored.

Table I : expander cohort's description:

28 expanders		
		<i>Implanted time (days)</i>
Allergan	17	228 (27 - 582)
<i>rupture</i>	2 (15)	325
<i>inflammation</i>	5 (12)	247 (27 - 536)
Mentor	11	271 (126 - 624)
<i>Rupture</i>	0 (11)	-
<i>inflammation</i>	4 (7)	443 (133 - 617)

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CAPSAICIN HELPS TO DECREASE COLLAGEN FIBRIL FORMATION AND IMPROVE THE STABILITY OF COLLAGEN FIBERS

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Keywords: Capsaicin, Type-I collagen, Tendons, Triple-helix, Collagen

INTRODUCTION

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is a versatile plant product widely used in foods and medicines. Topical application of capsaicin is known to have benefits against a variety of pain complications associated with such diseases as diabetic neuropathy and osteoarthritis. Other known health benefits of capsaicin involve its antihypertension, antitumorigenic and anti-inflammatory properties. Studies have proved that inactivation of capsaicin-sensitive nerve fibers reduces the pulmonary remodeling which occurs in collagen and elastic fibers and capsaicin treatment reduces the presence of collagen fibers in the vessels and lung tissues. To the best of our knowledge, direct studies of the capsaicin–collagen relationship are scarce in the literature. Hence, it is important to understand the effects of capsaicin on the biophysical properties of both the molecular and fibrillar forms of collagen. Collagens are structural proteins in the extracellular matrix which are crucial in providing the mechanical framework of many tissues, for example skin, tendons, and blood vessels. In this study we attempted to determine whether capsaicin has any effect on the biophysical properties of collagen.

RESULTS AND DISCUSSION

It was found that capsaicin suppresses collagen fibril formation, increases the stability of collagen fibers in tendons, and has no effect on the molecular stability of collagen. Turbidity assay data show that capsaicin does not promote disassembly of collagen fibrils. However, capsaicin moderately protects collagen fibrils from enzymatic degradation. Computational studies revealed the functions of the aromatic group and amide region of capsaicin in the collagen–capsaicin interaction.

We conducted collagenolytic degradation of capsaicin-treated rat tail tendons. The amount of hydroxyproline released from collagenase-treated RTT was measured to quantify the degradation of collagen fibrils in the presence of capsaicin. Approximately 99 % of the native collagen fibers were degraded in the absence of capsaicin. Capsaicin-treated RTT were, however, resistant to collagenolytic hydrolysis. Capsaicin–tendon interaction we measured the shrinkage temperature of RTT in the presence of capsaicin. Native collagen fibers the shrinkage temperature was ~ 56 °C, which is higher than the denaturation temperature of collagen triple-helical molecules (~ 41 °C). We observed an increase in the shrinkage temperature for collagen fibers pre-treated with capsaicin. The CD spectra of collagen in the presence and absence of capsaicin (Fig. 4a). In the presence of capsaicin, collagen molecules retained their inherent triple-helical conformation (a positive peak at ~ 222 nm), and we noticed an increase in the intensity of the peak at 222 nm in the presence of capsaicin.

CONCLUSIONS

Accumulation of collagen is one of the main causes for the onset of lethal diseases such as fibrosis and atherosclerosis. A straightforward approach targeting collagen fibril formation could therefore be critical for treatment of such diseases. This study revealed the potential of capsaicin to suppress collagen fibril formation. This unique property of capsaicin may have potential in the development of drugs for treatment of diseases linked with excess collagen fibrillogenesis.

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SMART HYDROGELS WITH ANTI-INFLAMMATORY PROPERTIES FOR BURN INJURIES

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Keywords: hydrogels, collagen, anti-inflammatory

INTRODUCTION

Hydrogels are three-dimensional structures that have the capacity to mimic human organs behaviour in terms of response to changes in environmental conditions such as pH, temperature, enzymes making them perfect for medical applications. Hydrogels can be used as dressings for moist healing of the wounds. It has the capacity to induce autolytic debridement, to absorb the exudate by expanding chains of crosslinked polymer. The high content of water allows vapour and oxygen transmission to the wounds. Hydrogels play a very important role in the emergency treatment of burns, alone or combined with other products due to its calming and moisturizing effect. Non-steroidal anti-inflammatory drugs are commonly used to reduce pain and inflammation associated with burn injury. Thus, the aim of this study was the development and characterization of some new composites based on collagen (COLL), polyvinyl alcohol (PVA) and Indomethacin (IND), designed to be used in thermal burn injury / wound healing.

MATERIALS AND METHODS

Type I fibrillar collagen gel was extracted from calf hide. Hydrogels with different concentrations of COLL and 5% PVA were investigated in terms of rheological analysis. The spongy composites, obtained by hydrogel freeze-drying, were evaluated by morphological (water-uptake, SEM), spectral (FT-IR) and biological analysis (enzymatic degradation) analysis. The *in vitro* IND release was carried out with a transdermal sandwich device adapted to dissolution equipment and the kinetic mechanism was set.

RESULTS AND DISCUSSION

The flow patterns recorded as viscosity versus shear rate for the designed hydrogels indicated a shear-thinning behaviour, and the rheological parameters specific to the Power law model were determined. The water absorption, enzymatic degradation and SEM analysis were correlated with the drug delivery profiles from spongy composites.

CONCLUSIONS

Based on the performance of the hydrogels and their lyophilized forms, the anti-inflammatory spongy matrices based on collagen and PVA could be promising samples for future applications in wound dressings for burn injuries.

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COLLAGEN-LIDOCAINE MICROCAPSULES WITH CONTROLLED RELEASE FOR TOOTH EXTRACTION PAIN

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Keywords: Microcapsules, Collagen, Analgesic

INTRODUCTION

The tooth extraction is associated with pain at several degrees of severity usually correlated with the amount of tissue damage involved in the procedure. The pain management is therefore of paramount importance and the local anesthetic treatment has to be considered. Topical drug administration is a reliable strategy ensuring a good patient compliance and a prolonged drug localized released effect. Thus, the purpose of this study was to design and characterize some collagen microcapsules, using as an anesthetic model drug the lidocaine hydrochloride.

MATERIALS AND METHODS

Type I collagen hydrolysate was obtained by acidic hydrolysis. The microcapsules were obtained from collagen hydrolysate cross-linked with tannic acid, genipin and glutaraldehyde, in various concentrations, and loaded with 1% lidocaine, followed by spray-drying. The size distribution, the morphology (SEM, swelling ability), the spectral characteristics (FT-IR), the goniometry (contact angle) of the designed microcapsules

were studied.

RESULTS AND DISCUSSION

Depending on the type of cross-linking agents different structures of collagen microcapsules were obtained. Their sizes and forms determined by SEM and zetasizer showed the spherical microstructure and size between 20 and 500 μm . The interactions between collagen and cross-linking agents were highlighted by FT-IR and the hydrophilic properties by contact angles and water absorption ability. Moreover, the crosslinking agents influence the release of lidocaine from collagen microcapsules.

CONCLUSIONS

As means to manage post intervention pain, the use of the proposed analgesic collagen microcapsules with controlled release depending on crosslinking agent was proved to be a promising solution.

ACKNOWLEDGEMENT

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EVALUATION OF BIODEGRADABLE NERVE CONDUITS BLENDED WITH TRADITIONAL CHINESE MEDICINE ON NERVE REGENERATION

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Keywords: Nerve regeneration, Biodegradable nerve conduit, Traditional Chinese Medicine, Berberine

INTRODUCTION

A nerve bridge technique is the introduction of both ends of the injured nerve stumps into a tubular chamber, which can offer the advantages of minimizing invasion and scarring of the nerve. Biodegradable nerve conduits can avoid the damaging problem of re-operation after embedding the conduit. Application of blending traditional chinese medicine and biomedical material to nerve regeneration is a new approach.

RESULTS AND DISCUSSION

This study evaluated peripheral nerve regeneration using a biodegradable nerve conduit, which was composed of glutaraldehyde cross-linked gelatin and blended with

traditional chinese medicine (Berberine) (Figure 1). In the cell culture test, we found that the berberine could be an effective accelerator for Schwann cells proliferation. The amount of berberine released from biodegradable nerve conduit was analyzed by Spectrophotometer and the test extended for 20 days at least (Figure 2). Histological observation showed that more axons and schwann cells had crossed through and beyond the gap region after 7 weeks of implantation.

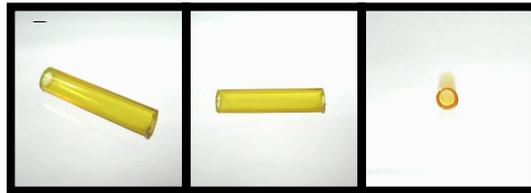


Figure 1: Biodegradable nerve conduit which was blended with berberine

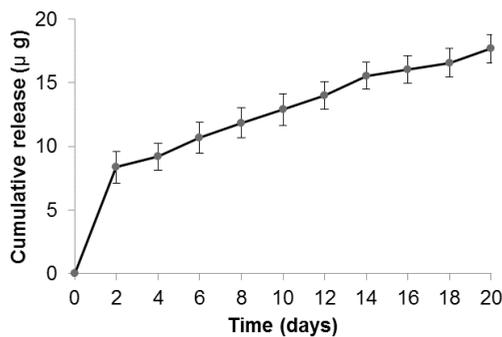


Figure 2: The amount of berberine released from biodegradable nerve conduit

CONCLUSIONS

Our data demonstrates that superiority of the biodegradable nerve conduits blended with traditional chinese medicine and not only be an effective aids for regenerating nerves but can also lead to favorable nerve functional recovery.

NANO GEL IN ONCOLOGY: A PROMISING CONTRIVANCE IN CANCER THERAPY

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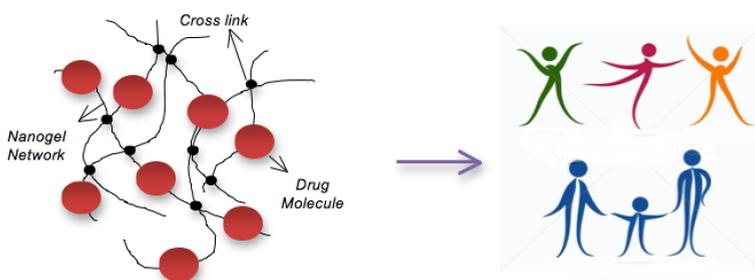
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Keywords: Nanotechnology; Cancer; Nanogel; Drug Delivery; Treatment.

INTRODUCTION:

Nanotechnology based nanogel approach is rising promising field of cancer management igniting the new scientific developments and permit clinical paraphrase beyond what we currently consuming. Nanogels have extended extensive cognizance in recent years as one of the most capable nano-sized drug delivery systems. Polymeric nanogel network have decent drug loading capacity, biodegradability, biocompatibility and optimum swelling ability which makes summit



RESULTS AND DISCUSSION:

This study imitates the recent expansion of nanogel drug delivery system in terms of variety of cancer treatment as a promising haulier system. Nanogel matrix system laden with bio-actives offers a wide assortment of cytotoxic

drug delivery on the specific site also nanogel loaded with nanoparticulate system opens innovative era of cancer treatment on onsite drug delivery with exclusion of adverse effect of cytotoxic drug as well to the normal tissue. Biomedical applications like pH responsive system, control release, sustain release, ligand anchored, thermo responsive nanogel system expand this nano drug delivery to the zenith sky in the field of cancer management.

CONCLUSION:

Thus Nanotechnology based nanogel drug delivery system is proving as boon for effective treatment of the world most threatening disease called cancer.

CHITOSAN AND CHITOSAN/ HYDROXYAPATITE COATINGS PRODUCED BY ELECTROPHORETIC DEPOSITION ON NEAR- β TITANIUM ALLOY AND THEIR IMPACT ON BIOACTIVITY, CELL ADHESION AND PROLIFERATION

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Keywords: Ti-13Nb-13Zr alloy, electrophoretic deposition, chitosan, hydroxyapatite, osteoblasts

INTRODUCTION

Nowadays titanium alloys, especially those without potential harmful elements such as vanadium and aluminium, e.g. near- β titanium alloys, are regarded as the most promising biomaterials for dental surgery and implantology. Their main advantages are relatively low Young's modulus, improved corrosion resistance and biocompatibility with bone tissue [1]. However to enhance their bioactivity and osteointegration various types of coatings can be applied, e.g. by electrophoretic deposition (EPD) [2,3]. The aim of this study was to find out if surface modification with chitosan alone (Chit) and Chit with hydroxyapatite (HAp) has a positive impact on biological performance of near- β titanium alloy deposited by EPD.

MATERIALS AND METHODS

Chit (Aldrich, Poland) and HAp (10 nm in size [4]) were applied as coating components on a near- β Ti-13Nb-13Zr titanium alloy (13.5-13.7 Nb, 13.5-13.8 Zr, 0.05-0.06 Fe, 0.04 C, 0.01-0.02 N, 0.001 H, 0.11 O (wt %); Xi'an Saite Metal Materials Development Co., Ltd, China, sample further encoded as Ti). Chit coating was deposited by EPD (5-30 V, 1-4 min) from 2 g/l Chit solution in a mixture 0.5% vol. acetic acid and 50% vol. ethanol (sample Ti/Chit). To obtain Ti/Chit/HAp sample Chit solution was enriched with HAp nanoparticles (4 g/l) suspended in ethanol. Morphology, layer thickness and chemical analysis were investigated by SEM/EDX (FEI, Nova NanoSEM 450). MG-63 osteoblast-like cells were cultured on manufactured samples and on tissue culture polystyrene (TCPS, 24-well Nunclon plates) for 1, 3 and 7 days at 37°C under 5% CO₂. Initial cell density was 8000 cell/cm². At each time point metabolic activity of cells (resazurin reduction, Sigma-Aldrich) was tested on FLUOstar Omega, BMG Labtech. Live/dead test (calcein AM/propidium iodide) and staining for cytoskeleton and nuclei (phalloidin/DAPI) were performed under fluorescence microscope (Axiovert, Zeiss). To evaluate bioactivity the samples were incubated in simulated body fluid (SBF, according to Kokubo method [5]) for 3 weeks; SBF was changed twice a week.

RESULTS AND DISCUSSION

Pristine near- β titanium alloy surface was smooth with a fine acicular martensite morphology as shown earlier [6]. As expected the substrate contained Ti, Nb, Zr and traces of other elements such as C and O (Figure 1 a); C content mainly resulted from carbon coating applied to make the samples conductive for SEM/EDX studies. Ti/Chit surface was smooth, homogenous and continuous with a thickness of 350 nm (Figure 1 b) and it contained more C and O resulting from presence of chitosan. Ti/Chit/HAp was rougher and consisted of nanocrystalline HAp particles and their agglomerates embedded in the chitosan matrix and the thickness of the coating was 1.5 μ m (Figure 1 c).

Presence of HAp nanoparticles was confirmed by EDX analysis which showed appearance of picks related to Ca and P; Ca/P ratio was 1.5. Immersion in SBF resulted in creation of typical cauliflower-like hydroxyapatite deposits only on Ti/Chit/HAp (Figure 1 f). The HAp layer showed presence of: Ca, P and O, i.e. the elements found in calcium phosphates. Ca/P ratio was 1.5, i.e. similar to that of measured for Ti/Chit/HAp. Although Ca/P ratio for stoichiometric hydroxyapatite is 1.66, one can presume

that obtained deposits are low-crystalline HAp, which may be also formed *in vivo* thus presumably enhancing osteointegration. On Ti and Ti/Chit (Figure 1d, e) HAp formation was hindered.

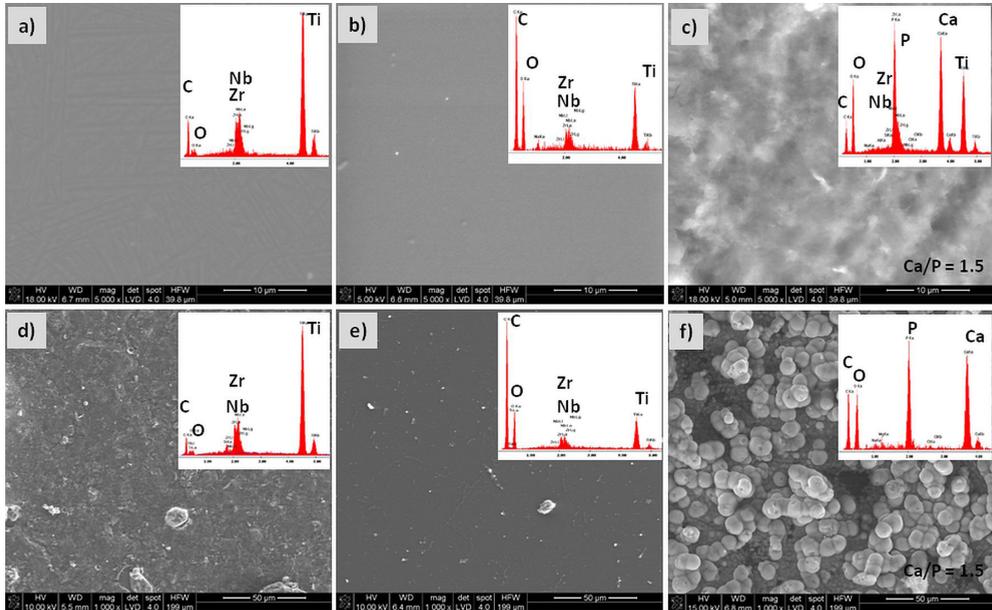


Figure 1: SEM/EDX analysis of: a) pristine near-β titanium alloy, and that coated by EPD with b) chitosan and c) chitosan/hydroxyapatite layer, d) pristine near-β titanium alloy after incubation in SBF, e) near-β titanium alloy with chitosan after incubation in SBF and f) near-β titanium alloy with chitosan/hydroxyapatite after incubation in SBF

In vitro tests show that Ti/Chit and Ti/Chit/HAp supported cell growth: cells adhering on the surfaces were alive (no red dead cells were found; Figure 2a panels 1 and 2), although the samples affected cell morphology, adhesion and proliferation in a different way. On day 1 cell morphology on Ti/Chit and Ti/Chit/HAp was similar: cells were round and less spread than grown on controls (Ti and TCPS). Their cytoskeleton fibers were poorly developed as compared to those on Ti and TCPS samples (phalloidin/DAPI stained cells - Figure 2a panels 3 and 4). On day 7 cells cultured on Ti/Chit/HAp were better spread, had polygonal shape and covered almost all available area in a way similar to control Ti and TCPS. Cell metabolic activity on day 1 and 3 was significantly impeded when the cells were cultured on Ti/Chit/HAp, however after 7 days no statistically significant difference

was found between controls (Ti and TCPS) (Figure 2b). On the other hand for Ti/Chit any difference was found on day 1, but for longer culture time (3 and 7 days) cell metabolic activity was negatively affected.

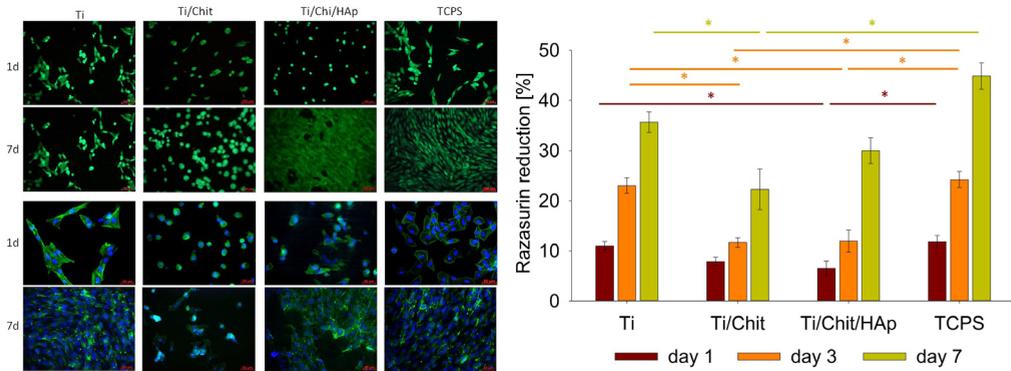


Figure 2: *In vitro* tests on MG-63 osteoblast-like cells cultured on pristine near- β titanium alloy (Ti), and that coated by EPD with chitosan (Ti/Chit) and chitosan/hydroxyapatite (Ti/Chit/HAp) and control tissue culture polystyrene (TCPS): a) live/dead staining (panel 1 and 2) and phalloidin/DAPI (panel 3 and 4) on day 1 (1d) and day 7 (7d); b) metabolic activity of cells after 1, 3 and 7 days

CONCLUSION

Chit and Chit/HAp coatings deposited on near- β Ti-13Nb-13Zr alloy by EPD were homogenous but the latter was more rough due to presence of hydroxyapatite nanoparticles and their agglomerates. On Ti/Chit/HAp thick layer of low-crystalline hydroxyapatite was created after incubation in SBF, what might suggest enhanced bioactivity of this surface that may be beneficial for osteointegration *in vivo*. Both coatings were not cytotoxic; Chit/HAp supported cell adhesion, proliferation and metabolic activity in a better way, especially for longer time points. Thus Ti/Chit/HAp seems to be promising for osteosynthesis, oral implantology and in joint prosthesis.

ACKNOWLEDGMENTS

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MORPHOLOGY, STRUCTURE AND CELLULAR RESPONSE OF THE RF MAGNETRON DEPOSITED ANTIMICROBIAL HAP-0.4ZN COATINGS ON TITANIUM

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Keywords: Hydroxyapatite, RF magnetron, TEM, XRD, Antimicrobial coating

INTRODUCTION

During the post-surgery period, a significant amount of antibiotics is used by patients in order to diminish the colonization of unwanted bacteria at the implant site [1]. Mechanisms of infection spreading are well known. In 90% of the cases, infection might get into the wound in exogenous manner. Other means of bacteria spreading causing infection shouldn't be forgotten, such as violation of sterility. Since an endoprosthesis surgery typically is performed in the open air, there will be always a risk of bacteria spreading to a wound during the surgery [2,3]. In order to effectively fight the infection, functional coatings on the surface of implants have been suggested. One of the possibilities is the formation of a hydroxyapatite ($(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2)$, (HAp)) coating doped with ions of certain metals (Ag, Zn, Cu and others) that are supposed to increase the bactericidal properties [4]. In this work nanocrystal Zn-doped (0.4 mol of Zn) hydroxyapatite coatings (HAp-0.4Zn) were deposited on the commercially pure titanium substrate by the RF magnetron sputtering method. Several regimes of the coating deposition with the variation of the substrate temperature during the sputtering process were done. Investigations of the coatings morphology, elemental

composition and structure were performed. Results of the biological tests including cytotoxicity tests and antimicrobial activity tests are presented.

RESULTS AND DISCUSSION

Structure of the coating underwent significant changes starting from the substrate temperature of 300°C. Coatings topography was presented by the structure elements with the average size of 260 nm for the samples which were heated to 200°C. The topography of the coatings surface is changing significantly for the samples deposited with the substrate temperature of 400°C. Structure changes are also proved by the Raman spectroscopy investigation. Intensity of ν_1 PO_4 vibration mode was significantly higher than the same mode intensity gathered from the sample deposited with the 200°C substrate temperature. That fact is telling about crystalline structure transformation in the way that the ν_1 PO_4 vibration mode became a favorable mode with regard to intensity. Dependencies of the substrate temperature during the deposition process on the coatings structure were discussed with regard to XRD, AFM and Raman spectroscopy results. For the cross-section transmission electron microscopy (XTEM) two methods of Focused Ion Beam techniques were applied. The XTEM analysis showed morphology and chemical composition of hydroxyapatite coating deposited at the standard conditions (magnetron power of 250W, sputtering duration of 3 hours, substrate temperature <50°C). Overview of the coating structure presented in the Figure 1.

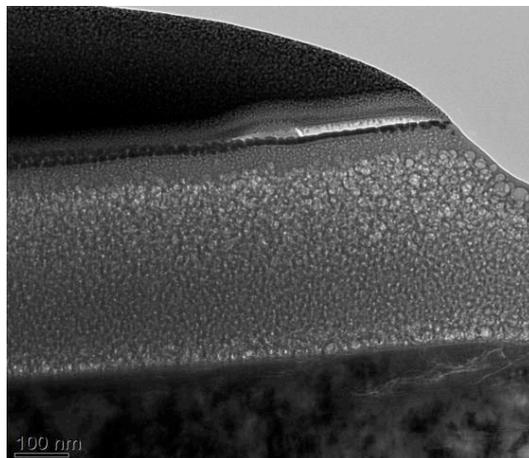


Figure 1: Structure and morphology of the HAp-0.4Zn coating deposited under standard conditions

The EDX analysis showed the presence of Zn of small concentration. The Ca/P ratio of the deposited coating was equal to 1.67 which is the same for stoichiometric hydroxyapatite. The amorphous hydroxyapatite sublayer of 8 nm thickness in the interface between crystal CaP coating and Ti substrate was detected. The HAp-0.4Zn coatings deposited with the standard conditions has a polycrystalline state with equiaxed grain structure. Cytotoxicity tests were performed using the mouse myoblasts C2C12 cell line. Tests showed absence of toxic effect. However, proliferation of the cells was decreased with the comparison to the same tests on the pure hydroxyapatite coatings. HAp-0.4Zn coatings showed bacteriostatic effect on the gram-negative e.Coli bacteria. Bacteria colonization on top of the HAp-0.4Zn coatings was significantly lower with comparison to Ti surface and HAp coating.

CONCLUSIONS

Coatings that were deposited under standard conditions and showed the presence of equiaxed grain structure with lattice planes orientation corresponding to the (122), (200) and (101) of HAp-0.4Zn. Interplanar d-spacing were calculated (2.39, 3.50, 4.07, 5.25 Å) and were the same with the standard values for hexagonal hydroxyapatite. Coating of HAp-0.4Zn was determined as a polycrystalline material with a thin amorphous layer (≈ 8 nm) in the interface between the coating and the substrate. The presence of the HAp in the coatings was confirmed with Raman spectroscopy by highlighting its characteristic peaks which are $\nu_1\text{PO}_4$ and $\nu_3\text{PO}_4$ vibration modes). There was significant intensity increase of the $\nu_1\text{PO}_4$ vibration mode in the coating that was deposited at the substrate temperature of 400°C. Biological tests showed absence of the toxic effect of the HAp-0.4Zn coating to the cells of C2C12 cell line. It is shown that the coating has bacteriostatic effect.

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COMPARATIVE INVESTIGATIONS OF THE STRUCTURE AND PROPERTIES OF MICROARC WOLLASTONITE-CALCIUM PHOSPHATE COATINGS ON TITANIUM AND ZIRCONIUM-NIOBIUM ALLOY

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Keywords: microarc oxidation, biocoating, calcium phosphate, wollastonite, titanium, Zr–Nb alloy.

INTRODUCTION

Titanium (Ti) and Ti alloys are widely used as both orthopedic and dental implants because of their excellent mechanical properties, high corrosion resistance, and biocompatibility [1, 2]. Zirconium (Zr) belongs to the same group as Ti in the periodic table and it shows similar mechanical and chemical properties. In addition, Zr shows unique properties that Ti does not have. For example, Zr does not form any calcium phosphate layer on it in a simulated body fluid [3]. There are many studies that involve the improvement of bioactivity of Ti, Zr and their alloys by the surface treatments. Microarc oxidation (MAO) is one of them [2]. It is a relatively new technique of the surface treatment based on anodic oxidation, which became famous for its ability to form in-situ grown porous and homogeneous oxide coatings on such metals as Ti, Al, Mg, Nb and their alloys. Additionally, it is one of the most effective methods to modify the surface of titanium and its alloys by the calcium phosphate coating formation for the best biocompatibility and bioactivity [1, 2]. Among the metals, MAO modification of Ti, Mg, and their alloys has been extensively investigated [4, 5], whereas studies on MAO modification of Zr and its alloys are still quite limited [6, 7]. The MAO process combines the electrochemical oxidation and the high-voltage spark treatment in

an aqueous electrolytic bath which also contains modifying elements in the form of dissolved salts to be incorporated into the resulting coatings [8]. To obtain oxide layers with higher concentrations of bioactive compounds, the MAO process can be performed in suspensions. The addition of hydroxyapatite, wollastonite, tricalcium phosphate, silica or other bioactive powders may enrich the coatings [9, 10].

This paper presents the results of comparative investigations of the structure and properties of wollastonite– calcium phosphate (W–CaP) coatings deposited by the MAO method on Ti and Zr–1.1wt. % Nb (Zr–1Nb) alloy.

RESULTS AND DISCUSSION

The influence of MAO parameters as oxidation voltage and process duration on morphology, thickness, roughness and adhesion strength of the W–CaP coatings was revealed. Figure 1 demonstrates SEM micrographs of W–CaP coatings deposited under different MAO parameters. Coatings with a thin calcium phosphate layer of 10–15 μm were formed under the low oxidation voltage of 130–150 V (Fig. 1 a, d) on Ti and Zr–1Nb.

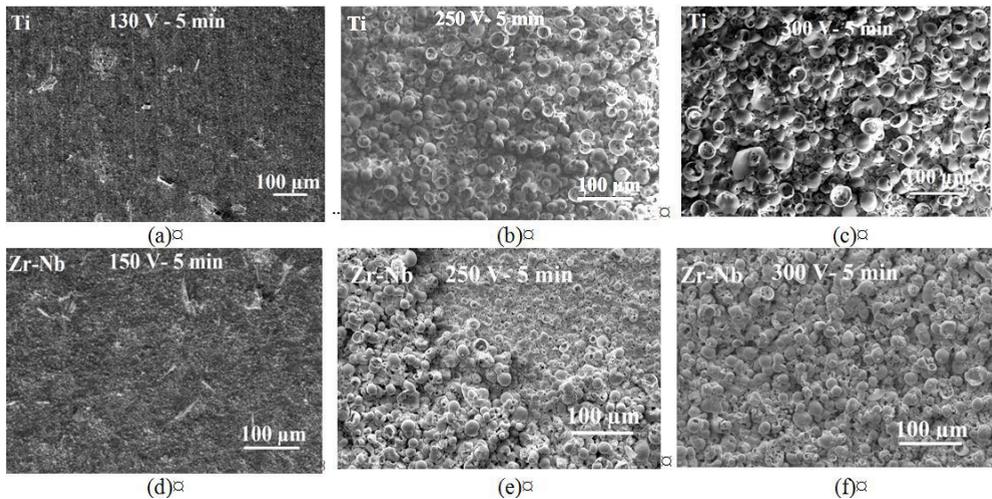


Figure 1: SEM images of the W–CaP coatings on Ti (a–c) and Zr–1Nb (d–f) produced during 5 min under different voltages.

A lot of wollastonite crystals with the size of 10–100 μm are observed in these coatings. Spherical structural elements (spheres) with pores are formed on the coating surface

under the oxidation voltage of 200–300 V (Fig. 1 b, c, e, f). Coatings deposited on Zr-INb under 250 V during 5-10 minutes have the surface inhomogeneity (Fig. 1 e). With the increasing of voltage from 250 to 300 V the surface inhomogeneity disappears and the coatings became uniform (Fig. 1 f).

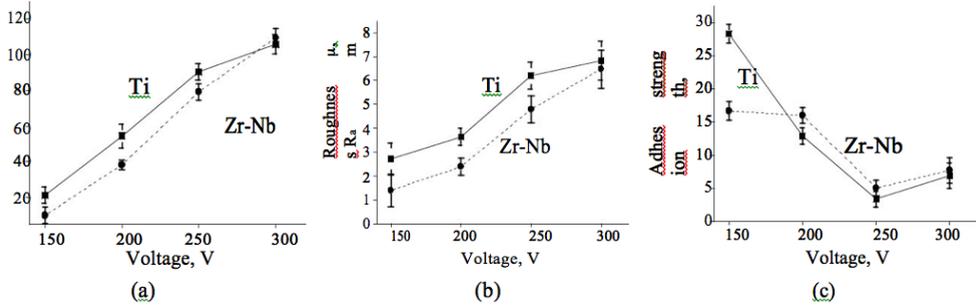


Figure 2: Graphs of the thickness (a), surface roughness (b) and adhesion strength (c) of W–CaP coatings on the Ti (a, c) and Zr–INb deposited for 5 min against the MAO voltage

Figure 2 shows the coating thickness, surface roughness and adhesion strength as the functions of oxidation voltage. The thickness of W–CaP coatings on both substrates increases linearly with the voltage increasing from 150 to 300 V (Fig. 2 a). This can be related to a current density growth and, as a consequence, to the increment of deposition rate of coating [8]. Also, the surface roughness of the W–CaP coatings on both substrates increases from 1.5 to 7.0 µm (Fig. 2 b). The maximum adhesion strengths are 28 and 16 MPa for coatings deposited under the applied voltage of 150 V during the 5 min process duration on the Ti and Zr–INb consequently. It is associated with the lowest thickness and roughness of such coatings. The increase of the oxidation voltage up to 300 V leads to the decrement of the coating adhesion strength up to 5 MPa [1].

CONCLUSIONS

Comparative investigations of W–CaP coatings on the Ti and Zr–INb alloy deposited by the MAO method under 150–300 V demonstrated that the processes of the coatings formation on both substrates were similar in many respects. It was clearly shown through identical linear dependences of the coating thickness and surface roughness on the process voltage. Coatings deposited on Zr–INb under the voltage of 150 V during 5 minutes have a small thickness and roughness. In this case the

recommended voltage range for the W–CaP coating deposition on the Zr–INb substrate is 200–300 V.

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RF-MAGNETRON SPUTTERED HYDROXYAPATITE COATING ON AZ91 MAGNESIUM ALLOY

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Keywords: Magnesium alloy, Biomaterial, Hydroxyapatite, Biodegradable implant, RF-magnetron sputtering

INTRODUCTION

The magnesium-based alloys are biodegradable biocompatible materials for bone joint replacement and they reveal similar mechanical properties with human bone. However, magnesium to be used as implant has some limitations such as a poor corrosion resistance in chloride-containing environment of the body. These implants can prematurely lose their mechanical properties before healing [1, 2]. A corrosion-resistant, protective coating on the surface of an implant is a promising way to achieve this challenge. It is known that calcium-phosphate (CaP) coating prepared by radio-frequency (RF) magnetron sputtering can improve a comprehensive range of future implant properties, e.g. high adhesion to the metallic substrate, good corrosion resistance and osteoinductivity [3, 4].

RESULTS AND DISCUSSION

The XRD patterns of the initial magnesium alloy AZ91 substrate and coated one, as shown in Fig. 1, indicate that the coating consists mainly of hydroxyapatite (HA) peaks and the peaks attributed to the substrate. No other high-temperature phases were

found, such as β -tricalcium phosphate (β -TCP; $\text{Ca}_3(\text{PO}_4)_2$), tetra-calcium phosphate (TTCP; $\text{Ca}_4(\text{PO}_4)_2\text{O}$), or calcium oxide (CaO), which can be formed in the plasma-spray process [5].

Tafel plots obtained from HA coated and bare AZ91 Mg alloy in simulated body fluid (SBF) are depicted in Fig. 1 (c). From the Tafel plots, it was observed that the corrosion potential (E_{corr}) of the uncoated alloy is quite negative (-1.54 V). In contrast, the corrosion potential of the coated alloy was more positive, shifting to about -1.52 V. It was also noticed that the corrosion current densities (i_{corr}) of the coated alloy was 0.35 mAcm^{-2} compared to that of the uncoated specimen, i.e. 80.9 mAcm^{-2} . Therefore, the potentiodynamic polarization test revealed that the corrosion resistance of the uncoated alloy was significantly improved

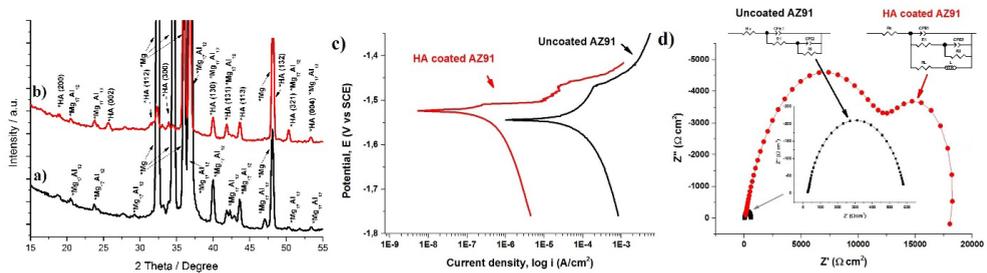


Figure 1: XRD patterns of uncoated AZ91 magnesium alloy (a) and HA coated AZ91 alloy (b). The determined lattice parameters were $a = b = 0.9452 \text{ nm}$; $c = 0.6952 \text{ nm}$, the average crystallite size was 25 nm . Electrochemical Tafel plots (c) and impedance spectra (d) of HA coated and bare AZ91 magnesium alloy in SBF solution.

The results of the impedance measurements showed (in Nyquist plot) the presence of 2 capacitive loops in the case of uncoated AZ91 specimens (Fig. 1, d). Therefore, the chosen equivalent electrical circuit for the fitting procedure consisted of two R/CPE components (a constant phase element (CPE) was used instead of a capacitor (C) to compensate the non-homogeneity of the electrode surface; Q and n are the CPE associated parameters). On the other hand, for all the HA coated samples, an inductive loop was observed at low frequencies, and based on these findings, a RL/L (resistor-inductor/inductor) component was added in the electrical circuit considered for fitting of the measured impedance data. Due to the noise present in the low frequency range, the fitting procedure was performed in the range: $0.05 \text{ Hz} \div 2 \times 10^4 \text{ Hz}$. The R_p of the coated alloy was found to be significantly higher compared with that of the bare alloy i.e., $R_p = 13700 + 6790 \Omega \text{ cm}^2$ for coated alloy,

and $R_p = 36.8 + 520 \Omega \text{cm}^2$ for bare alloy. Therefore, the HA coated AZ91 specimens became more corrosion resistant than uncoated AZ91 alloy.

The SEM images after electrochemical corrosion tests are shown in the Fig. 2 (a, b). It was observed large number of corrosion pits and localized corrosion attacks occurred much more severely in the case of uncoated AZ91 alloy. The comparison of the HA coated alloy results with the ones for the uncoated alloy indicated that corrosion resistance was improved by the deposition of the coating.

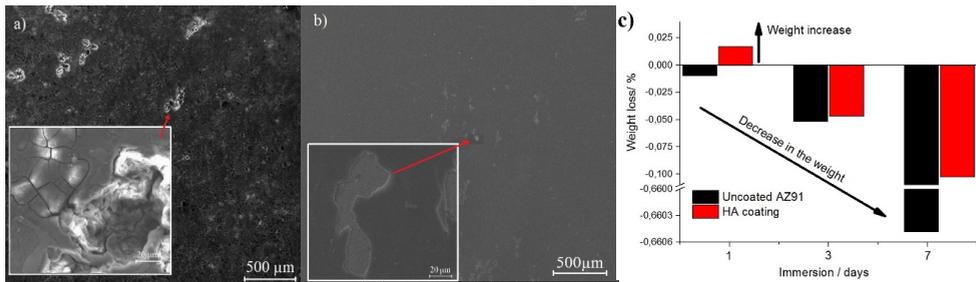


Figure 2: Corrosion morphology of bare (a) and HA coated (b) AZ91 magnesium alloy. Variation of the weight loss percentage of uncoated AZ91 and HA coated samples as function of immersion time in SBF (c).

Fig. 2 (c) shows results of the immersion test for the pure AZ91 and the HA coated alloy within different immersion periods (1, 3 and 7 days). The weight loss rate is presented in percentage. The uncoated alloy demonstrated a trend of the higher weight loss rate compared to the coated one. The weight loss rate measured after a seven-day immersion of AZ91 is 7 times higher than that of the coated sample. A comparison of the immersion test between the AZ91 and the HA coated alloy indicated that corrosion resistance was improved by the application of the HA coating.

Parameters	AZ91 alloy	HA-coated AZ 91 alloy
Water contact angle, °		
101 ± 6		
51 ± 3		
Free surface energy, mN/m	22 ± 2	46 ± 3
Polar component, mN/m	1.2 ± 0.1	32 ± 4
Dispersive component, mN/M	21.6 ± 2.2	14 ± 2

Table I. Wettability parameters of the uncoated and HA coated AZ91 magnesium alloy

Investigations revealed improvement of the surface wettability of the HA coated samples with water ($51 \pm 3^\circ$) compared with the bare alloy ($101 \pm 6^\circ$). Based on the obtained results for a set of different test liquids, an increase in the surface energy (from 22 ± 2 to 46 ± 3 mN/m) and, in particular, an increase in the polar component of the free surface energy (from 1.2 ± 0.1 to 32 ± 4 mN/m) was observed.

CONCLUSIONS

Thus, in this study AZ91 alloy was coated with HA by means of RF magnetron sputtering. The HA coating reduced the corrosion current density (i_{corr}) of the alloy from 80.9 to 0.35 μA and significantly enhanced the polarisation resistance (R_p) (by a factor more than thirty times) under *in vitro* conditions. The weight loss measurements of the coated samples in simulated body fluid showed a 7 times decrease compared to the bare alloy. This observation clarifies the ability of the HA coating to decrease its corrosion rate. To summarize, the obtained results allow us to conclude that a layer of HA deposited on the surface of AZ91 alloy allow to control its degradation rate.

ACKNOWLEDGEMENTS

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MAGNETIC ASSEMBLING OF FIBROBLASTS INTO THE CORD-LIKE STRUCTURE

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Keywords: Cellular cord; Magnetically labeled cells; Magnetic field; Cell viability; Cell assembling

INTRODUCTION

Magnetically labeled cells have been proven to respond to and be moved easily by a magnetic field to create simple biomimetic structures with a potential utility in tissue engineering. Dragging by external magnetic field of cells loaded with magnetic nanoparticles promotes solutions inaccessible to other methods in particular for creation of cell chains. Here, we go beyond these approaches by making use of suitably applied magnetic fields sufficiently strong to orient and accumulate the magnetized cells in 3-dimensional 150 μm thick cellular cord. The magnetic assembling of cells gives a possibility to organize a large number of cells in elongated ordered structures along the lines of magnetic field.

RESULTS AND DISCUSSION

It has been shown that $10^5 - 10^6$ MNP (5-50 pg/cell) are able to readily move a cell by magnetic gradients of 0.1-1 T/m. Noteworthy, this amount of MNP does not exceed 0.1-1% respectively of the cell volume and it does not alter any of the investigated cell properties. Normal human fibroblasts (Lonza, CC-2511) were cultivated in T-75 culture flasks with advanced DMEM/F12 (Invitrogen) supplemented with 15% FBS (Bioclot), 0.1 mg/mL streptomycin sulfate and 100 U/mL potassium penicillin, 2mM glutamine in humidified atmosphere of 37°C and 5% CO₂. The medium was changed completely every two to three days. Once cells reached subconfluence, the culture

medium was supplemented with polyaspartic acid coated fluorescent nano-screen MAG/G-PAA nanoparticles (Chemicell, green fluorescence) and incubated overnight. Then cells were rinsed with PBS, detached with trypsin/EDTA (0.025/0.02%) and concentrated by centrifugation at 200 g. Thereafter MSCs has been dissolved in abovementioned medium and placed in rectangular transparent cuvette with following hermetic sealing using Parafilm. Following manipulation has been realized using couple cylindrical magnet with IT magnetization as it demonstrated on the Figure 1. assembled cells have been oriented by the magnetic drive into the fibrillar structures with following development of homogeneous cell cord.

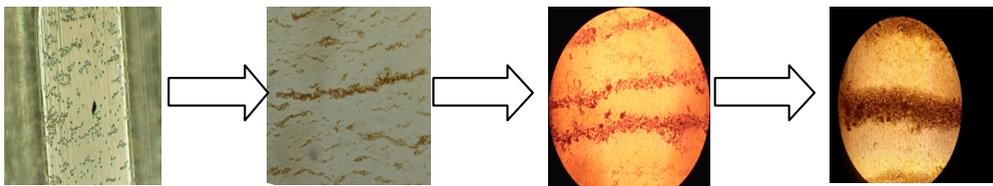


Figure 1: Cell cord development from separate magnetically labeled fibroblasts.

CONCLUSION

We believe that the manipulation of magnetically labeled cells using spatially oriented magnetic field represents a unique solution for building complex cell assemblies organized in a biologically adequate arrangement and in particular for generation of in vitro tendons/ligaments rudiments, which will need to develop and mature after implantation.

MAGNETO-SENSITIVE MULTI-BLENDED CRYOGELS FOR HARD TISSUE ENGINEERING

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Keywords: magnetic nanoparticles, collagen, TCP, cryogels, tissue engineering

INTRODUCTION

In order to regenerate or repair a tissue due to trauma or congenital defects; researchers may use biomaterials/scaffolds to support growth and to allow new tissue formation. There are several techniques for scaffold preparation such as solvent casting, molding, electro-spinning, wet spinning, and rapid prototyping. In addition, several natural or synthetic polymers such as poly glycolic acid, poly lactic acid, poly ε-caprolactone and even silicon have long been used for the construction of the scaffolds [1]. Collagens based scaffolds, alone or in blended forms; are mostly prepared in hydrogel or in membrane forms and used for wound dressings, cell encapsulation, or controlled release studies.

Cryogels, on the other hand is rather a new forms in tissue engineering and can be form by cryogelation technique which based on the forming a hydrogel below the melting point of the solvent. The initial studies on cryogels are mainly formed from synthetic polymers and used for chromatographic applications and separation techniques. However, there has an existing interest of using biopolymeric cryogels in biological and medical applications [2].

In a physiological point of view, cells should interact both with each other and the ECM substrate for proliferation, differentiation and carrying out their functional duties. Therefore, a scaffold should also have the ability to effect cell behaviour by either mimicking the ECM topography and composition or by generating physical/mechanical stimuli to induce the cells. Mechanical stimuli can be generated by applying external physical forces via a designated instrument such as bioreactors or can be

generated via specialized particles. Magnetic particles are one of them. Magneto-sensitive scaffolds can be achieved by the combination of magnetic nanoparticles, bare or coated with a functional polymeric shell, with polymeric scaffold to form a biohybrid biomaterial.

Here, magneto-sensitive collagen (Col)-carboxymethyl cellulose (CMC) cryogels were prepared for possible use in hard tissue engineering.

RESULTS AND DISCUSSION

Super-paramagnetic particles were synthesized by co-precipitation method from its Fe^{+2} and Fe^{+3} salt precursors [3]. Further analyses were performed by Zeta-Sizer and Vibrating Sample Magnetometer (F1). The results showed that particles has $\leq 90\text{nm}$ size (by light scattering method) and have around 1.315 Tesla magnetic strength.

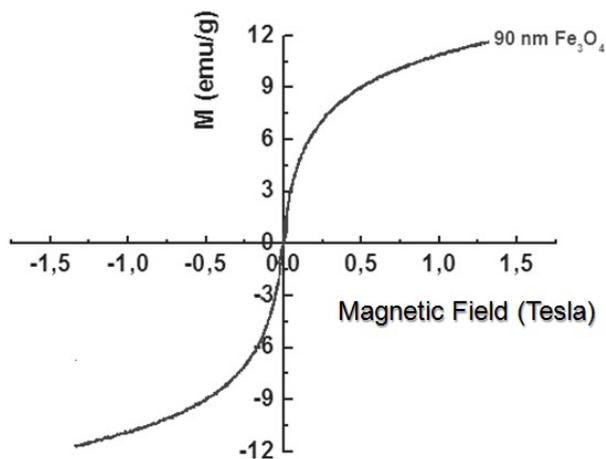


Figure 1. VSM of magnetic nanoparticles

On the other step; magneto-sensitive cryogels were prepared by the modification of a previous report [4]. Briefly, rat tail tendon collagen was swollen in 0.1% Acetic acid (w/v). CMC solution was prepared from its sodium salt in distilled water. Air dried magnetic particles were mixed with the Col/CMC solution (1% (w/w)) After 4 hours, solutions was poured into polystyrene wells and kept under -20°C for 48hours and later on samples were lyophilized for 24hours. Lyophilized cryogenic samples were cross-linked via well-known carbodiimide chemistry with EDC/NHS. Cryogels were then kept under -20°C for about 24hours and later on, samples were lyophilized

once again for 24hours (F2). Further chemical and structural characterization were performed by Fourier transform infrared spectra (FTIR), thermogravimetric analysis (TGA), X-ray crystallography (X-RD) and scanning electron microscopy (SEM). Mechanical properties were tested by unconfined compression test. Moreover, hemocompatibility of the cryogels were also evaluated by basic biochemical blood testing. Chemical and structural analysis results demonstrated the well achievement of the cross-linking without any major alteration in collagen and carboxymethyl cellulose with a thermally and structurally stable blend formation. Scanning Electron Micrographs demonstrate the multi-lamellar formation with macro and micro pore composition which can correlate with water uptake results of the cryogels. Hemocompatibility evaluations exhibited that the cryogels are non-toxic and blood-compatible.

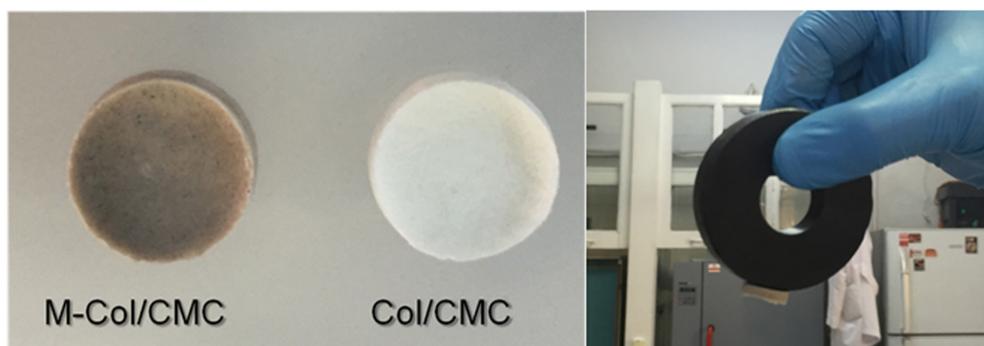


Figure 2. Magneto-sensitive Col/CMC cryogels

CONCLUSIONS

The overall results shows that these magnetic particle consisting collagen carboxylmethylcellulose cryogels may have potential use as a material for hard tissue regenerations as they have both the ability to induce the cell proliferation and growth and ability to generate a proper physical stimuli throughout the cells.

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DEVELOPMENT OF NOVEL APPROACHES FOR TUMOUR THERAPY BASED ON NANOSTRUCTURED MATERIALS - MAGBIOVIN PROJECT

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Keywords: MagBioVin, magnetic hyperthermia, radiolabeling, magnetic nanoparticles, core shell.

Research advancements and opportunities by the FP7-ERA Chairs project MagBioVin are spotlighted.[1] Topic of the project is the design of different novel magnetic nanoarchitectures (e.g. bimagnetic and polymeric core-shell systems, nanoparticles embedded in mesoporous silica structures, and radiolabeled nanostructures)[2–4] for application in targeted treatment and diagnostics of cancer. These nanomaterials possess the ability for selective treatment of tumor tissues by the targeting with magnetic field.[5,6] Alternating magnetic field also provides the means for hyperthermia-induced cancer treatment.[7] Attachment of radionuclides to the synthesized nanoparticles is explored for the purpose of imaging and internal radiotherapy.[8,9] Magnetic characteristics of the prepared nanomaterials is done by SQUID magnetometry and Mössbauer spectroscopy. Structural characterization of the investigated nanomaterials is performed by XRD, TEM imaging, DRIFT spectroscopy, and nitrogen sorption analysis. Magnetic hyperthermia effects are monitored by using commercial setup (nB nanoScale Biomagnetics) which includes applicators for cell cultures and small animals. In vitro and in vivo (animal model) applicability of the synthesized nanomaterials regarding toxicity, biodistribution and anti-cancer efficacy is explored for targeted cancer treatment.

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MAGNETIC NANOPARTICLES IN BIOMIMETIC BIOPOLYMER -CALCIUM PHOSPHATES COMPOSITES FOR BONE TISSUE REGENERATION

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Keywords: magnetic nanoparticles, bone regeneration, biopolymers, calcium phosphates

INTRODUCTION

The bone has a high regenerative capacity, therefore the majority of the fractures, especially in the case of young people, heal without a surgical intervention. The difficulties arise in the case of bone tumours [1]. In majority of the cases the bone tumour treatment implies two steps. The first step involves the surgical resection of the tumoral tissue and the second step is represented by the filling of the obtained bone defect with a bone graft material. Various types of materials based on natural and synthetic polymers, mixed or not with ceramics have been developed. Scaffolds with inclusion of magnetic nanoparticles are increasingly studied as bone graft materials for bone tumours [2].

Magnetic nanoparticles are most often used for magnetic resonance imaging (MRI) as contrast agents and for cancer therapy (hyperthermia) as heating mediators [3]. In bone tissue engineering, functionalized magnetic nanoparticles are used as drug delivery systems providing controlled release of bioactive molecules (growth factors, anti-tumoral drugs or associated molecules) [4].

The current study presents the obtaining and characterization of scaffolds based on

biopolymers, calcium phosphates and magnetic nanoparticles in the aim to apply them in bone tissue regeneration.

MATERIALS AND METHODS

Calcium phosphate precursors (calcium chloride - CaCl_2 and monosodium phosphate $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) have been precipitated on a polymeric matrix containing three of the most used biopolymers in tissue engineering (collagen, chitosan and hyaluronic acid), in presence of magnetic particles. The precipitation process mimics the bone nanostructure, an essential requirement of the scaffolds used as bone substitutes [5]. The morphology of the scaffolds was studied using Scanning Electron Microscopy. The chemical structure was studied using Fourier Transform Infrared Spectroscopy (FTIR) and X-Ray Diffraction (XRD). *In vitro* enzymatic degradation, retention of simulated body fluids and *in vitro* biocompatibility studies have been performed in order to evaluate the biological performances of prepared scaffolds.

In vitro biocompatibility studies. After sterilization, samples of the magnetic scaffold have been incubated in a humidified incubator (37°C , under 5% CO_2) for 24 hours in DMEM medium containing 10% bovine fetal serum and 1% penicillin/streptomycin/neomycin (P/S/N). In the same conditions, preosteoblast cell line MC-3T3 were incubated for 24 hours. Cells were seeded at a density of 10^4 cells per well in 24 well plates. After the incubation period, the samples of the scaffolds were placed in direct contact with osteoblast monolayer for 72 hours. At 48 hours and 72 hours MTT assays have been performed.

RESULTS AND DISCUSSION

The magnetic scaffolds and the MNPs have been analyzed by using FTIR analysis and peaks from the three biopolymers and the calcium phosphates are present. The three-dimensional porous structure of the scaffolds (Figure 1) influenced the retention of simulated body fluids. The retention degree of simulated body fluid is attributed especially to the three-dimensional porous structure of the magnetic scaffolds and the hydrophilic properties of the three biopolymers. *In vitro* enzymatic degradation is influenced by the composition of the scaffolds. Good results have been obtained for the *in vitro* biocompatibility studies.

Scaffold degradation is a very important issue because throughout the degradation period, various properties can be damaged. The influence of the Ca/P ratio on the

degradation of the scaffolds was observed to be less important than the concentration of biopolymers in the initial mixture. The advantages of biodegradable comparing with non-biodegradable materials is the disappearance of implanted material, which might hold off the foreign body reactions of the body defensive system over a long-term contact with a living tissue.

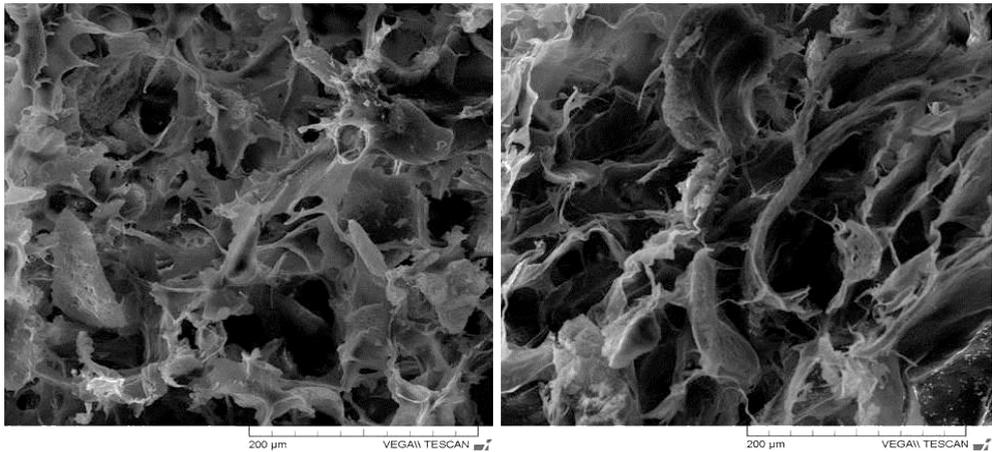


Figure 1: SEM data of scaffolds (left – high content of calcium phosphates, right – low content of calcium phosphates)

The metabolic activity of the viable cells has been measured at the 24 h, 48 h and 72h and the values of the absorbance have been measured in the aim to calculate the cells viability. When the cells are viable in all culture means that the scaffolds have good citocompatibility. The obtained results indicate that the ratios between polymers monitor de cell – material interactions.

CONCLUSIONS

Scaffolds based on magnetic nanoparticles, calcium phosphates and biopolymers have been obtained and characterized. The three-dimensional porous morphology of the scaffolds influences the retention of simulated body fluids and enzymatic degradation, which is also influenced by the Ca/P ratio and the content of polymeric phase. The physico-chemical and biological characterization indicates that the scaffolds exhibited several properties required for bone tissue engineering.

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ENZYMES-BIOFUNCTIONALIZED MAGNETIC NANOPARTICLES FOR CARDIOVASCULAR APPLICATIONS

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Keywords: magnetic nanoparticles, enzymes, biofunctionalization, cardiovascular applications

INTRODUCTION

Some diseases of the cardiovascular system are associated with increased production of reactive oxygen species (ROS), and few treatments of the vascular tissue disorders involve antioxidant enzymes [1]. The efficiency of enzyme-based treatment is dependent by the ability to achieve therapeutically adequate levels at the site of ROS-mediated injury [2]. Enzymes-biofunctionalized magnetic nanoparticles can increase the enzyme efficiency and limits adverse effects of thermal and proteolysis degradation [3]. In terms of application, such magnetic nanoparticles have to be stable in the circulating system and to improve the half-time of the biological compounds, to reach the diseased tissue or to be involved in the in cellular mechanisms, to release the bioactive in the controlled manner, activated or not by external stimulus/stimuli. Also, they have to be biodegradable and eliminated from body without any toxic effects [4]. Moreover, in the aim to attach biomolecules on surface, the magnetic nanoparticles have to present reactive groups, like us: OH and NH₂, COOH and SH, most often obtained by coating with

polymers. Various polymers have been tested as coatings or networking structure in composites with magnetic materials (PLA, PLGA and copolymers, other synthetic polymers, chitosan, hyaluronic acid, gelatine) [4].

This paper presents the biofunctionalization of magnetic nanoparticles based on magnetite and poly (maleic anhydride-co-3,9-divinyl-2,4,8,10-tetra-oxaspiro (5.5) undecan) erythritol with two antioxidant enzymes (catalase and superoxide dismutase (SOD)). These enzymes contain active centers with coordinated metals, which decompose superoxide anion and hydrogen peroxide, respectively, the most important reactive oxygen species [5]. Magnetic material selected for this study was the trivalent iron oxide, Fe_3O_4 (magnetite). Obvious benefits are the excellent magnetic properties that allow manipulation in magnetic field, superparamagnetic behavior at nanoscale dimensions and biocompatibility. These structures have been coated with a copolymer based on maleic anhydride-undecane-erythritol. Maleic anhydride is a biocompatible monomer and its functionality allows the reaction with various molecules (drugs, proteins, enzymes). Undecane is a biodegradable monomer and erythritol is a polyol attached in the purpose of opening the maleic anhydride ring, has antioxidant properties and minimizes the effects of endothelial dysfunction [6].

MATERIALS AND METHODS

Biofunctionalized magnetic nanoparticles with antioxidant enzymes have been obtained by Catalase/SOD attaching on particles through carbodiimide chemistry method. Briefly, 5 mg enzyme was dissolved in 20 mL phosphate buffer saline (PBS, 0.05 M, pH=8.00) and then mixed with a suspension of nanoparticles (100 mg in 30 mL of 0.05 M PBS, pH=8.00) and slowly mechanically stirred for 24 h. The particles have been collected from suspension through magnetic separation, purified by repeated washes with PBS (2x) and deionised water (3x) and finally freeze-dried.

The chemical structure was studied using Fourier Transform Infrared Spectroscopy (FTIR) and dimension by dynamic light scattering (DLS), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Zeta potential and redispersion in simulated body fluids have been performed in order to evaluate the possibility to be formulated as injectable fluids.

In vitro biocompatibility studies. Cytotoxicity tests have been performed after sterilization and incubation for 24 hours in DMEM medium containing 10% bovine fetal serum and 1% penicillin/streptomycin/neomycin (P/S/N). In the same conditions, fibroblasts were incubated for 24 hours. Cells were seeded at a density of 10^4 cells per

well in 24 well plates. After the incubation period, the samples of the scaffolds were placed in direct contact with cells monolayer and MTT assays have been performed at 24h, 48 h and 72 h. For hemocompatibility tests, the blood was collected by venous puncture from healthy volunteers and was incubated with an anticoagulant (sodium citrate solution, 3.3%; ratio 1/9 v/v). In 3 ml of blood was added a suspension of nanoparticles (0.5 ml; 0.01%) and incubated at 37 ° C for 1 h and 12 h (under slight agitation). Finally, the particles were separated by centrifugation (1000 rpm, 10 min) and the blood was biochemical analyzed. The study was approved by the Ethics Committee of the Grigore T. Popa University of Medicine and Pharmacy I asi.

RESULTS AND DISCUSSION

The FT-IR data, zeta potential and hydrodynamic particles mean diameter (dynamic light scattering evaluation) confirmed the both enzymes immobilization onto magnetic nanoparticles. In the FT-IR spectra, the amide bond formed between the enzyme and the copolymer is evidenced by changes in the characteristic bands, the peaks at 1567 cm^{-1} and 1618 cm^{-1} . The particles morphology, analyzed by electron microscopy (SEM) and transmission electron microscopy (TEM) suggests that some clusters are formed during the freeze-drying process.

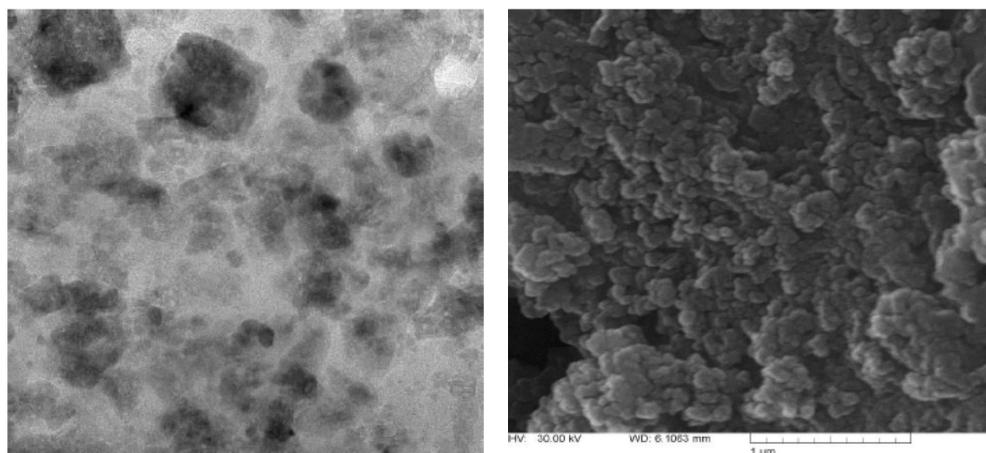


Figure 1. SEM (left) and TEM (right) data of enzymes-biofunctionalized magnetic nanoparticles

Redispersing studies were performed in different environments: water, PBS (phosphate buffer), FBS (biological fluid simulated), FBS with albumin (1%) and the

glucose solution 3%. It was noted that the best dispersion medium is 3% glucose solution; particles with average diameters under 400 nm and a polydispersity index of 0.480, which proves increased suspension stability. Enzymatic activity of magnetic nanoparticles has been determined indirect by NTB-methionine-riboflavin (in the case of superoxide dismutase) and direct, with H₂O₂ (for catalase). All tests on the enzymatic activity of nanoparticles functionalized with both enzymes indicated that the enzyme activity is maintained; nanoparticles concentration, the enzyme nature and the biological molecule conformation onto magnetic nanoparticles have important influences on the enzymatic activity.

The MTT assay revealed the citocompatibility of the enzyme-immobilized magnetic nanoparticles, cells viability being more than 90% after 72 h, and it was comparable with the control; the cell morphology and their proliferation are not affected by the magnetic nanoparticles. The hemocompatibility tests showed normal values for the concentration in haemoglobin, hematocrit, red blood cells, leukocytes and platelets. Also, the values obtained for the prothrombin time and fibrinogen were within normal limits after 1 h of incubation of the nanoparticles with blood.

CONCLUSIONS

Enzymes-biofunctionalized magnetic nanoparticles have been obtained by SOD/catalase immobilization onto nanoparticles based on magnetite and poly (maleic anhydride-co-3,9-divinyl-2,4,8,10-tetra-oxaspiro (5.5) undecan) erythritol. The biofunctionalized magnetic nanoparticles are redispersable in biological-friendly medium, biocompatible and exhibits enzymatic activity which recommend them in cardiovascular applications.

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SAXS/WAXS MICROSCOPY OF 3D COLLAGEN-BASED ENGINEERED TISSUES FOR NERVES AND VESSELS REGENERATION

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Keywords: SAXS, WAXS, collagen, nerves, vessels, engineered tissues

INTRODUCTION

One of the most important goal in the fabrication of engineered tissues is the replacement of injured tissues with functional biological substitutes. Collagen-based biomaterials represent a class of materials successfully used to support the regeneration, because of their biocompatibility, biodegradability and mechanical properties [1]. 3D collagen-based tissues, engineered for nerves and vessels regeneration, have been morphological and structural characterized by X-ray scattering techniques (SAXS/WAXS). The experiments were carried out at the XMI L@b [2-3-4] (CNR-IC-Bari). Structural properties were combined with relevant biological and chemical features to inspect and fully exploit the biomaterial functionalities [5]. Wide-angle X-ray scattering transmission microscopy (WAXS) allowed an atomic structural analysis. Small-angle X-ray scattering (SAXS) analysis warranted a detailed characterization of the tissue-engineering scaffolds at the nanoscale.

RESULTS AND DISCUSSION

Engineered tissues for nerves - Wide-angle X-ray scattering transmission microscopy (WAXS) analysis on different raw collagen matrices (Collagen Solution, Kensey-Nash, TypeOneCH, TypeOneE and Symatase), used for nerves regeneration, showed the presence of two collagen specific strong reflections: equatorial diffraction peak ($q = 0.58 \text{ \AA}^{-1}$, $d = 10.8 \text{ \AA}$) that reflects the distance between the molecular chains of collagen; meridional peak ($q = 2.21 \text{ \AA}^{-1}$, $d = 2.8$) that

reflects the distance between adjacent amino acid residues along the central axis of collagen helical structure. This is the evidence that in all collagen matrices the helical structure is preserved after extraction processes. However, matrices have a different degree of crystallinity. 2D profiles of TypeOneCH and TypeOneE (equine tendon collagens) showed preferred orientation on both equatorial and meridional reflections.. Analysis of equatorial peak of each collagen (Figure 1 (i.)) showed an increase of crystallinity from Kensey-Nash, Symatese and Collagen Solution, to TypeOneE and TypeOneCH. Indeed, Kensey-Nash and Symatese (bovine dermis collagen) show more amorphous-like profiles, and Collagen Solution seems to be less pure than the other matrices containing sharp reflections which do not belong to the collagen structure.

Engineered tissues for vessels - Small-angle X-ray scattering (SAXS) analyses allowed nanostructural characterization of 3D scaffolds made of collagen alone and Human Elastin-Like Polypeptides (HELP)-enriched collagen for vessels regeneration, compared with pure collagen, pure HELP and native aorta vessels, used as reference model. Figure 1 (ii.) shows that collagen and collagen-HELP matrices profiles have many common features and almost the same periodicity of the porcine vessel's one. SAXS profiles of 3D scaffolds made of collagen alone and HELP-enriched collagen appear dissimilar from those acquired from samples of pure collagen and pure HELP. Moreover the results show an increase in the similarity degree between native porcine aorta model and artificial collagen-HELP scaffolds with the incubation time.

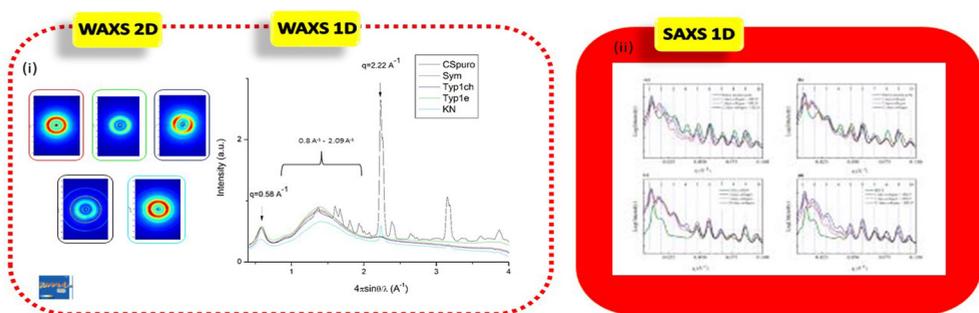


Figure 1 . (i) WAXS data (2D images and profiles) for different raw collagens used in engineered scaffolds for nerve regeneration (ii). SAXS profiles after background subtraction and deconvolution comparison between (A) the collagen scaffolds and native porcine vessel, (B) the collagen-HELP scaffolds and native porcine vessel, (C) the collagen scaffolds and pure collagen sample, and (D) the collagen-HELP scaffold and pure HELP sample.

CONCLUSIONS AND/OR OUTLOOK

WAXS analysis allows us to inspect atomic structural differences among different raw collagen used for nerves regeneration, such as different degrees of crystallinity and orientation, depending on native tissues and extracting processes. These differences were found to influence the overall scaffold. Nanoscale SAXS analysis on scaffolds used for vessel engineering showed a hierarchical organization of each collagen matrix, which was related to the biomechanical properties of the scaffolds [5].

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TABLE TOP SCANNING SAXS/WAXS MICROSCOPY OF BIOMATERIALS

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Keywords: SAXS microscopy, X-ray microdiffraction, hydroxyapatite, interfibrillar packing

INTRODUCTION

We show the application of a table top super-bright microfocus laboratory X-ray source coupled to a three-pinhole camera (XMI-L@b [1,2]), which was used to investigate the nano-micro structure of biomaterials in SAXS/WAXS ex-situ experiments. The combination of the microsource brilliance and statistical/crystallographic approaches allowed us to restore diffraction features from SWAXS profiles collected from different kinds of materials or soft tissues, reaching data quality compared to synchrotron radiation data [3]. As relevant examples we will show SWAXS data obtained through a scanning microscopy method applied to: biomimetic scaffolds for bone tissue regeneration [4], bovine cornea [5] and peptide-based fibers [6]. Our results show how precious morphological/structural nanoscale or atomic information can be gained by mapping biomaterials and tissues.

RESULTS AND DISCUSSION

The first step for an in depth understanding of the function/structure relation of a material is certainly a detailed structural characterization at all hierarchical levels. The presented test cases have been selected from relevant research fields in order to provide representative examples of biomaterial characterization performed by the X-ray Microlmaging Laboratory (XMI-L@b) facility [1,2] (Figure 1A) and the crystallographic software developed at IC-CNR [3].

- SAXS/WAXS - An investigation of gelatin scaffold filled with a gradient of

hydroxyapatite (30wt%) for bone tissue engineering application has been successfully performed at XMI-L@b [4]. Microdiffraction experiments combining SAXS and WAXS analysis, allowed us to ascribe the absorption gradient, revealed from intensity transmission measurements, to the presence of a gradient in the concentration of HA (SAXS) and to confirm that HA is the only crystalline phase in the sample (Figure 1B).

- SAXS - The second relevant example concerns a bovine cornea which underwent to the UV-CXL (cross-linked) treatment procedure. The cornea was studied by SAXS scanning microscopy at both synchrotron (SLS-cSAXS) and laboratory (XMI-L@b) beamlines [5]. Comparable results were found revealing: *i*) a decrease in the interfibrillar distance and in the shell thickness around the fibrils from the periphery to the center of the cornea (the central area coincides with the region where the epithelium has been removed for the CXL treatment); *ii*) no significant change in the diameter of the fibrils was measured across the explored area; *iii*) the array of the fibrils resulted packed according to a centered hexagonal symmetry (Figure 1C).
- WAXS - Peptide self-assembled materials, based on PEGylated-tetraphenylalanine fibers, were studied by WAXS, in presence and in absence of a DOTA chelating agent [6]. Aim of the study was here to understand how the aggregation process influences the performance of the nanostructures used as innovative contrast agent in magnetic resonance imaging. WAXS measurements indicate an antiparallel β -sheet organization of the monomers in the resulting fibers. Moreover, WAXS experiment points out that in solution the nanomaterials retain the same morphology and monomer organization of the solid state, although the addition of the DOTA affects the size and the order degree of the fibers (Figure 1D).

CONCLUSIONS

SAXS and WAXS techniques were applied, at the XMI-L@b, in different fields of biomaterials/tissue analysis to explore their supra-molecular and/or sub-molecular structure.

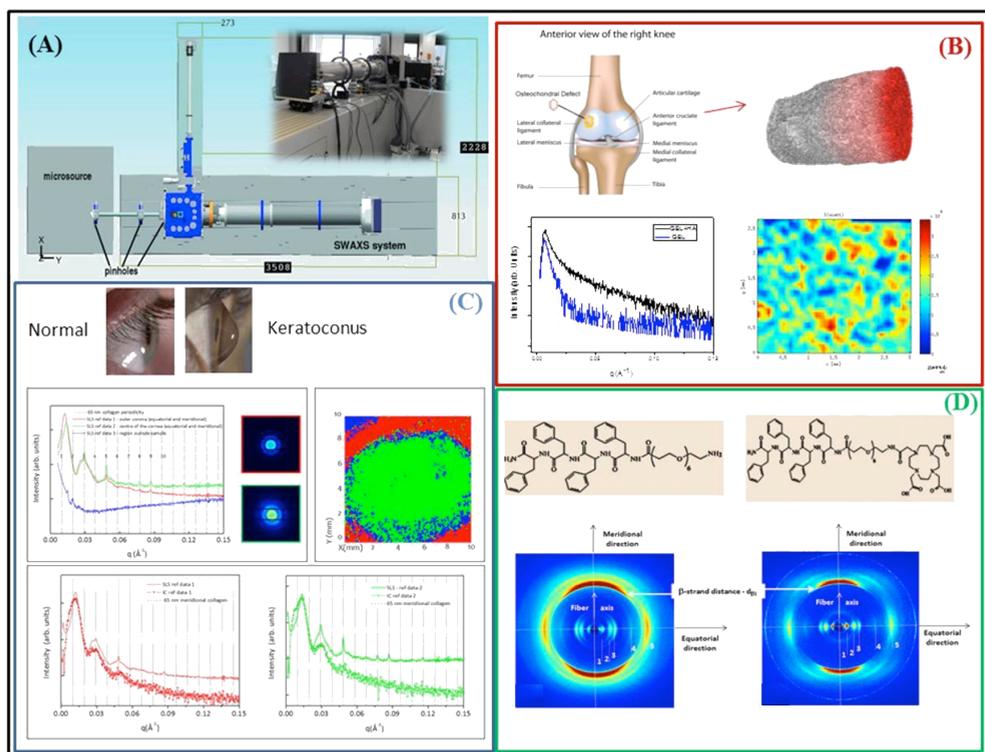


Figure 1: XMI-L@B equipment (A), microscopy of gelatin/HA scaffold for bone tissue engineering (B), study of interfibrillar packing of bovine cornea (C), structural characterization of peptide-fiber as potential contrast agent in MRI application (D).

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