Sulfated alginates as biomaterials

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INTRODUCTION:

Alginates show promise for biomaterials in tissue engineering and slow-release systems due to their favourable gelling conditions, inherent biocompatibility and high stability *in vivo*. Alginates are relatively inert and generally do not interact with cells or proteins to promote adhesion, migration and proliferation, which has led to the exploration of various functionalization strategies.[1] In addition, the monosaccharide sequence of alginates can be customized *in vitro* using C-5 epimerases as a tool to tailor gel properties.[2] Chemical sulfation of alginates confers an analogous molecular structure to sulfated glycosaminoglycans (GAGs), which are major components of the extracellular matrix with vital roles in cell signaling related to tissue growth and homeostasis.[3] Through the studies described here, we aimed to investigate whether sulfated alginates have similar functions as sulfated GAGs, and if they can be utilized in novel biomaterials with tailored chemical and biological properties.

METHODS:

Homogeneous sequences of sulfated alginates were prepared by chemoenzymatic modification of bacterial alginates, and structurally characterized, to study protein-binding properties. To characterize their effect on cells and evaluate their potential in tissue engineering, sulfated alginate hydrogels were prepared and used to cultivate chondrocytes in 3D. Anti-inflammatory properties were assessed by applying both soluble and cross-linked sulfated alginates in a whole-blood model and through inflammatory induction of encapsulated chondrocytes.

RESULTS:

Sulfated alginates were found to interact with growth factors (HGF, FGF) similarly to heparin in a sulfation degree- and monosaccharide sequence-dependent manner. Chondrocytes encapsulated in sulfated alginate gels showed a high degree of proliferation and spreading, (Fig. 1) and were found to retain their chondrogenic phenotype while suppressing markers of de-differentiation and catabolism. Furthermore, sulfated alginates demonstrated anti-inflammatory properties by inhibiting cytokine expression, complement and leukocyte activation in blood, and suppressing IL-1β-mediated inflammation in chondrocytes.

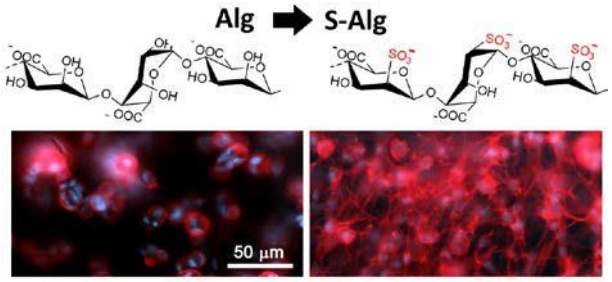

Fig. 1: Phalloidin/DAPI staining of human chondrocytes cultivated in unmodified alginate gels (left) and in sulfated alginate gels (right).

DISCUSSION & CONCLUSIONS:

Sulfated alginates demonstrate great promise for cartilage engineering, and potential for cultivation of other tissues, by serving as a reservoir and co-receptor for growth factors. Additionally, their anti-inflammatory properties can help reduce host responses to implanted biomaterials and improve integration of engineered tissues. Sulfated alginates have a tuneable sulfation degree and monosaccharide sequence to allow control over the molecular structure, which is limited in naturally occurring GAGs. These features can aid in the elucidation of structure-function relationships, and allow tailoring of biological and physical properties of the hydrogels for specific applications.

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3D cell culture of adherent cells within alginate and alginate-collagen hydrogels

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INTRODUCTION: Currently there is much interest in developing techniques to culture cells in 3D within a hydrogel matrix. This is because hydrogels may inherently, or be engineered to, mimic physicochemical aspects of the extracellular matrix. This has implications in the fields of cell and molecular biology, tissue engineering and pharmaceutical development. Significant material challenges arise from this approach, which are primarily derived from limitations in physical structuring techniques and intrinsic biomaterial-cell interactions.

Recently, we described a new method of controlling the kinetics of crosslinking ions to gel ionotropic polymers, termed Competitive Ligand EXchange (CLEX).[1] CLEX has proven advantages for the cytocompatible gelation of alginate across a variety of length scales, including gelation within microfluidic channels.[1,2] In our previous work, several cell types were encapsulated within CLEX gels and survived for prolonged periods in culture. However, adherent cells were unable to interact with and spread within the alginate matrix due to the inert nature of the biopolymer. Here we have taken two approaches to overcome this limitation, firstly by employing an alginate modified with RGD peptide (RGD-Alg) and secondly by combining alginate with collagen type 1 to create a composite hydrogel (Alg-Col).

METHODS: CLEX crosslinked alginate hydrogels were prepared as described previously.[1,2] For Alg-Col composites, 1% type 1 collagen was combined with 0.8% CLEX alginate prior to gelation. MC3T3-E1 pre-osteoblasts or fibroblasts were encapsulated in 6 µL discs as described previously[3] or encapsulated in microbeads using a dual aqueous flow microfluidic biochip.[2]

RESULTS: Adherent cells grown within CLEX crosslinked gels appeared to attach to the hydrogel matrix in both RGD-Alg and Alg-Col composites and then spread within the gels. Cell growth was monitored up to 7 days, upon which time significant cell spreading could be observed (*Fig 1A*). Fibroblast cells were successfully encapsulated in RGD-Alg microbeads prepared by droplet based microfluidics with high viability (92%) and cells began to attach to the hydrogel matrix after 24hrs in culture (*Fig 1B*). No significant cytotoxic effects were observed for either preparation up to 7 days in culture by means of alamarBlue® and LDH assays (data not shown).

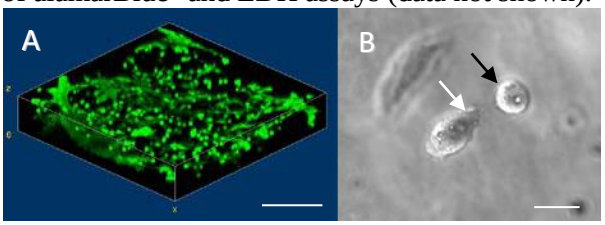

Fig. 1: A: Pre-osteoblast cells cultured within an Alg-Col gel after 7 days, stained with calcein AM. Scale bar = 2 mm. B: Fibroblast cells encapsulated in an RGD-Alg microbead in early stage of attachment showing one cell beginning to adhere (white arrow) compared to a rounded non-attached cell (black arrow). Scale bar = 10 µm.

DISCUSSION & CONCLUSIONS: Here we have demonstrated the application of a new crosslinking mechanism for ionotropic hydrogels to enable 3D culture of adherent cells. This was possible by either modification of the alginate polymer to include RGD peptide motifs, or by combining unmodified alginate with collagen. By incorporating collagen we have demonstrated the concept of using CLEX to template the formation of a second hydrogel present within the composite that gels by other means. Initial experiments indicate excellent cytocompatibility for both approaches and adherent cells were able to spread within both hydrogel matrices. Further work is needed to fully characterise the fate of both the cells and the hydrogel structures, particularly in terms of cell differentiation and material integrity respectively.

ACKNOWLEDGEMENTS: The authors acknowledge financial support provided by the Norwegian Research Council through FRINATEK project No. 214607 (PS) and FORNY project No. 260700 (DCB).

Biomimetic polysaccharide/peptide amphiphile supramolecular multilayered nanobiomaterials as instructive cell platforms

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INTRODUCTION: To date, several supramolecular systems have been developed aiming at mimicking the complex composition, structure, dynamic and functional behavior of the native extracellular matrix (ECM).[1] Although very promising, such systems still lack control in thickness, composition and structure, and the functional dynamic nature and structural complexity found in the biological systems. Herein, we highlight the fabrication of biomimetic supramolecular multilayered nanobiomaterials comprising negatively charged high-molecular-weight alginate (ALG) and oppositely charged low-molecular-weight self-assembling peptide amphiphile (K_3PA) molecules, through combination of self-assembly and electrostatically-driven layer-by-layer (LbL) assembly approaches. Alginate was used to trigger the self-assembling capability of positively charged K_3PA molecule. The LbL technology was further used to fabricate, for the first time, supramolecular multilayered nanobiomaterials with controlled composition, structure and function by repeating the deposition of both molecules.

METHODS: The buildup of $(ALG/K_3PA)_5$ nanofilms was monitored by quartz crystal microbalance with dissipation monitoring (QCM-D). The morphological properties were investigated by atomic force microscopy (AFM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). *In vitro* biological performance was assessed using C2C12 cells.

RESULTS: TEM (Figure 1a) and SEM (Figure 1b) revealed the nanostructured properties and 1D nanofibrous network of the assembly formed by both molecules. The C2C12 cells behavior was significantly affected by the nature of the outermost layer. Enhanced cell behavior was observed on the supramolecular systems having K_3PA as outermost layer (Figure 1c,d).

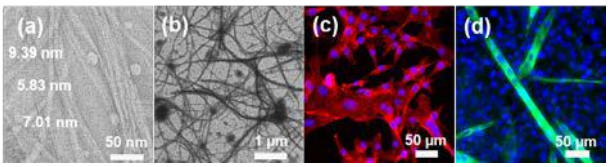

Fig. 1: (a) TEM image of the ALG/K_3PA (1:1, v/v ratio) nanofibrous network. (b) Morphological characterization of $(ALG/K_3PA)_5$ nanofilms by SEM. Representative (c) fluorescence microscopy and (d) immunofluorescence micrographs of C2C12 cells at 3 and 5 days of culture, respectively, on $(ALG/K_3PA)_5$ nanofilms.

DISCUSSION & CONCLUSIONS: The newly developed supramolecular multilayered nanobiomaterials revealed a nanofibrillar structure which emulates the structural and functional features of the native ECM. Myogenic differentiation studies showed that K_3PA-ending films led C2C12 cells to differentiate into multinucleated myotubes with a tube-like morphology, proving their myogenic potential. The proposed supramolecular multilsyered nanobiomaterials have great potential to be used as promising bioinstructive matrices for biomedical and healthcare applications.

ACKNOWLEDGEMENTS: JB, SGC and MPS acknowledge the financial support by the Portuguese Foundation for Science and Technology (FCT) through the grants SFRH/BPD/103604/2014, SFRH/BPD/96797/2013 and SFRH/BD/97606/2013, respectively. This work was also supported by ERC grant agreement ERC-2014-ADG-669858 for project "ATLAS".

Unravelling the potential of free-standing membranes from natural-based polymers made by layer-by-layer for biomedical applications

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INTRODUCTION: Although tissue engineering (TE) has witnessed tremendous progresses in recent years due to their unlimited potential to improve human health, it is still facing several challenges in order to conceive adequate tools for the repair of tissues. TE offers a promising alternative strategy of healing severe injuries by utilizing the natural biological response of the body to tissue damage in conjunction with engineering principles. Cells, growth factors (GFs), and biomaterial scaffolds form the foundation of most TE strategies. Moreover, lessons from nature pinpoint that mimicking the complexity and hierarchical organization of the tissues can offer advantages for the success of TE strategies. Thus, a good understanding of the tissue organization from molecular level up to the macroscopic scale and understanding how the different components interact with one another will serve as a good guide for the rational design of the artificial substitute. In this context, building artificial structures in a bottom-up assembly seem a good strategy. A bioinspired approach that may be able to create tissue-like structural complexity by assembling blocks mimicking the tissue units in a bottom-up is the Layer-by-Layer (LbL) methodology. LbL enables the fabrication of films with supramolecular nano- to micrometric stratified architectures. This is achieved by sequential adsorptions of oppositely charged polyelectrolytes onto a large variety of substrates. The popularity of such method is due its user-friendly preparation, possibility to incorporate various biomolecules, fine control over the film architecture and robustness of the production under ambient and physiological conditions. Moreover, LbL allows the incorporation of a wide range of materials, from synthetic to natural polymers, proteins, or even other bioactive molecules. In the last years LbL has been transposed towards the production of free-standing (FS) membranes. The production of FS is quite simple: like in the supported LbL films the deposition is made onto a substrate. The difference lies that the assembly do not remain attached to the substrate. FS membranes are able to keep the relevant properties of LbL films and they also allow the direct measurements of several physico-chemical and mechanical parameters, including ion or biomolecule permeation and mechanical properties that is impossible to measure when the LbL film is attached to a substrate. Such parameters are particularly important if the intent is to design new materials membranes to be tuned for real-world applications. In this context, valuable information can be achieved not only about the surface but also to the bulk composition of the FS. Such FS membranes can possess gradient properties or even distinct characteristics through their thickness. For instance, the stiffness of the FS membranes can be tuned in order to promote cell adhesion [1] and they can even present reversible switchable properties. [2] Moreover, FS membranes can act as reservoirs for GFs. [3] More recently, nanopatterned FS membranes were produced to control cell alignment and morphology by redesigning ECM-mimetic.

In this work, several examples of FS membranes will be presented where their potential for biomedical applications will be disclosed. We particularly highlight the potential of using natural-based polyelectrolytes in the preparation of such nanostructured devices.

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Dual tumor targeting therapy on breast cancer cells by doxorubicin-bioconjugated carboxylic acid magnetic nanoparticles

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INTRODUCTION: Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide, accountings for 25% of all cancer cases and 15% of all cancer deaths among females [1]. The aim of the present study was to produce a dual targeted drug delivery system and magnetic hyperthermia for more effective treatment of breast cancer by using carboxylic acid functionalized iron-oxide nanoparticles (CA-SPIONs - Fe_3O_4) loaded with doxorubicin which were able to be localized in target tissues and internalized within the desired cells by application of an external magnetic field [2].

METHODS:

The electrostatic loading of doxorubicin (DOX) to SPIONs was achieved through the electrostatic interaction between the carboxyl group of carboxylic acid functionalized iron-oxide nanoparticles and the amine group of DOX. After formation of the drug-nanoparticle conjugates, they were separated from the free drugs via magnetic separation and washing in the aqueous medium.

The therapeutic effect of DOX-CA-SPIONs was evaluated by MTT assay on the following cell lines: MCF-7 (Human breast cancer cells ER+/PR+), Triple negative MDA-MB-231 breast cancer cells, MCF-10A (Mammary epithelial cells). In this way we tried to verify the selectivity of magnetic hyperthermia treatment induced by SPIONs on killing breast cancer cells respect to normal mammary epithelial cells.

For hyperthermia treatment, cells were put in the incubator at 46°C for 30 minutes, corresponding to a temperature dosage of 90 cumulative equivalent minutes at 43°C, or else cells will be left in the incubator at 37°C. At 48 h after hyperthermia, cells viability will be tested. The in vitro efficacy was also tested without hyperthermia treatment.

RESULTS: The cytotoxicity of DOX loaded CA-SPIONs was compared with free drug DOX in the absence and presence of magnetic field, also the probable cytotoxicity of the targeted and non-targeted blank nanoparticles were checked. Cell viability assay showed that in MCF-7 and MDA-MB-231 cells the cell survival percentage was decreased significantly ($p<0.05$) in targeted nanoparticles group compared to free DOX and naked magnetic nanoparticles in most drug concentrations both in absence and presence of magnetic field without toxic effects on normal mammary epithelial cells (MCF-10A). These data indicate that DOX-CA-SPIONs and the presence of magnetic hyperthermia led to increasing cytotoxic effects of the nanoparticles on breast cancer cells.

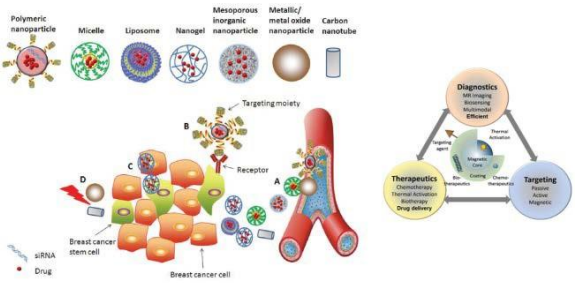

Fig. 1. Various approaches explored to target breast cancer using nanomedicines.

DISCUSSION & CONCLUSIONS: The results demonstrated the potential of the DOX-CA SPIONs to achieve dual tumor targeting by magnetic field-guided in breast cancer cells. This may reduce the required dose of DOX and consequently reduction the side effects of this drug. The results should be checked in vivo to confirm the promising results obtained from the cell culture tests. We discovered an intrinsic therapeutic effect of magnetic nanoparticles on cancer growth of breast cancer cells.

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Design and *in vitro* evaluation of bare and silica-coated iron-oxide nanoparticles

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INTRODUCTION:

Superparamagnetic iron oxide nanoparticles (SPIONs) have recently been investigated for biological applications with promising results, owing to their ability to be targeted and heated by magnetic fields [1]. Among all types of nanoparticles, biocompatible superparamagnetic iron oxide nanoparticles (SPIONs) with proper surface architecture and conjugated targeting ligands/proteins have attracted a great deal of attention for drug delivery applications [2]. Silica is a very suitable coating material for SPIONs, facilitating the loading of targeting moieties and drug delivery. However, the potential toxicity of SiO_2-coated SPIONs remains a major concern for clinical application.

METHODS: The synthesis, via wet-chemistry, and physico-chemical characterization of Fe_3O_4 and silica coated (Fe_3O_4-SiO_2) SPIONs are here described, examining *in vitro* cytocompatibility including viability, necrosis, intracellular reactive oxygen species (ROS) generation and apoptosis, in an endothelial cell model.

RESULTS: The results showed that both types of SPION are spherical, 10-15 nm in diameter and can be dispersed in water-based media. *In vitro* characterization revealed both to be highly cytocompatible at 10 μg/ml concentration, suggesting their safe use in biomedical applications. Cytotoxicity, including ROS generation and expression of apoptosis activating enzymes (caspase 3), slightly increased to 80 μg/ml, in a dose dependant manner. Fe_3O_4-SiO_2 nanoparticles induced a higher level of ROS and expression of caspase 3.

DISCUSSION & CONCLUSIONS:

In this study, Fe_3O_4 and Fe_3O_4-SiO_2 SPIONs were synthesized and characterized from the morphological, compositional, structural and biological standpoints. Particles were spherical, with diameters in the 10-15 nm range, dispersed, and stable in aqueous solution. These data suggest potential biomedical applications for both SPION types. The present study opens the door to explorations into the aspects of drug delivery and gene therapy, using SPIONs coupled to antibodies, siRNA and non-viral or viral vectors. Moreover, the surface of iron oxide NPs could be modified by organic materials or inorganic materials, such as polymers, biomolecules, metals, etc. The problems and major challenges, along with the directions for the synthesis and surface functionalization of iron oxide NPs, were considered. However, research in this field has great perspectives and numerous issues were optimized to be used safely this approach for targeted therapies and cancer therapy.

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Attachment and morphology of human mesenchymal stem cells on PEG-maleimide hydrogels

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INTRODUCTION: The extracellular matrix not only provides physical anchorage to cells, but also plays an important role in migration, proliferation and differentiation (1, 2). Mimicry of the extracellular matrix can prove effective in regulating cellular attachment and tissue regeneration. The purpose of this study is to assess changes in attachment and morphology (i.e actin organization, cell spreading, solidity and roundness, focal adhesion formation) of mesenchymal stem cells (hMSCs) on poly-ethylene glycol maleimide (PEG-MAL) hydrogels functionalized with increasing concentrations of RGD peptides. Additionally, we seek to identify the minimum amount of RGD necessary for hMSC attachment on these gels while documenting changes in hydrogel elasticity.

METHODS: Sterile protease sensitive 4-arm PEG-MAL hydrogels functionalised with linRGD and cycRGD (0.05, 0.5, 1.5 and 2.5 mM) were synthesized as adapted from Lutolf et al (3). Circular gel disks were formed between glass slides coated with dichloromethylsilane and a 1 mm spacer. Disks were placed in NUNC™ chamber slides and swollen in mesenchymal stem cell growth media (MSCGM™). $5x10^3$ hMSCs ($P \leq 7$) cells were seeded directly onto the hydrogels. Control setups included a PEG hydrogel lacking RGD functionality, cells cultured on glass coverslips and an 8-arm PEG gel functionalised with 2.5 mM cycRGD. Alexa® Fluor 568 phalloidin was used to fluorescently label the cytoskeleton and DAPI (1:1000) for the nuclei. Additionally, focal adhesion formation was assessed using primary mouse-anti human vinculin antibody along with a secondary antibody conjugated to Alexa® Fluor 488. Atomic force microscopy will be used to correlate mechanical changes and RGD concentrations within the gels.

RESULTS: Mature focal adhesions were formed in hMSCs cultured on glass cover slips. However, all 4-arm gels functionalised with RGDs showed only cytosolic expression of vinculin within the hMSC cell body and no distal localization of vinculin at the end of the actin filaments. 8-arm PEG-MAL controls show some vinculin expression. Additionally preliminary tests reveal only minimal cell penetration within the gel over 30 days.

DISCUSSION & CONCLUSIONS: Though focal adhesions form on stiffer substrates (4, 5), the threshold at which hMSCs form mature focal adhesions is not explicitly defined. This is difficult to outline due to the complex dynamics of focal adhesion assembly and the multiple factors contributing to its formation. Our initial results indicate focal adhesion formation on stiffer gels and glass substrates via vinculin expression, however it is important to further verify this via immunolabelling of multiple proteins within the focal adhesion complex. Additional measurements of cell spreading, circularity and focal adhesions are required to understand any implications the substrate may have on cell morphology. Concurrently, the mechanical changes with increased RGD functionality will elucidate the relationship between gel stiffness and cell morphology.

Nanohydroxyapatite synthesized by using microwave energy for medical applications

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INTRODUCTION: Bioactive materials which can support bone ingrowth and osseointegration are common used in orthopedic and dental applications. Hydroxyapatite (HAp) is a calcium phosphate compound having a chemical formula $Ca_{10}(PO_4)_6(OH)_2$. It is an inorganic component of hard tissues such as bones and teeth, which is responsible for strength and stiffness. Our bioactive hydroxyapatite (GoHAP^{TM}) can be obtained by the microwave reactor and the high pressure consolidation technology for ceramic materials.

METHODS: Nanohydroxyapatite was synthesized by precipitation method in room temperature and hydrothermal synthesis using microwave reactor MSS2. The morphology, grain size and specific surface area of the nanopowder can be controlled by the microwave reactor [1][2]. Obtained nanoparticles were in the range of 8 - 45 nm grain size. Phase purity was measured using X-ray diffraction. Thanks to scanning electron microscopy (SEM) the morphology of produced nanohydroxyapatite was characterized. The density and specific surface area were determined using helium pycnometry and BET method.

In the Laboratory of Nanostructures from Institute of High Pressure Physics (IHPP PAS) we are able to synthesize and obtain six types of hydroxyapatite with different crystallinity degree and grain size. Our GoHAp has a high similarity to the natural mineral bone.

RESULTS: Our projects with GoHAP^{TM} powders:

The aim of the **GoIMPLANT** project was to develop resorbable, tough, strong and biocompatible hybrid composite implants in according to patient's needs by using the isostatic and uniaxial pressing. To get better mechanical properties in our laboratory we used combination of GoHAP^{TM} and biocompatibile polymer like polylactic acid (PLA). Biodegradable composites can be decomposed naturally after a certain period of implantation.

The **SONOSCA** project innovation was to use the sonochemical process to coat polymer scaffolds with resorbable HAp and nano-TCP. Such coatings could increase the scaffold biocompatibility.

The main goal of the **iTE** project is to develop a novel scaffold-based in vivo tissue engineering approach to regenerate large bone defects in oncological patients.

NanoLigaBond project is about to set up a GMP approved production line for GoHAP^{TM} powder and using the semi-automatic sonochemical reactor for coating method of porous and fibrous materials with nano-particles of GoHAP^{TM}.

AntiBacTex is about to develop textiles with antibacterial and bacteriostatic properties without release chemicals into the environment. The work will be brought to the stage of commercialization the product

ACKNOWLEDGEMENTS: Research was realized by the "GoIMPLANT" Project (2013-2016) and it is founded by M-Era.Net program of The National Centre for Research and Development, co-financed from the European Union, Regional Development Fund.

Extracellular matrix as a template for hard tissue engineering

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INTRODUCTION: The extracellular matrix of all tissues and organs is a highly organized and complex structure unique to the specific organ type. The ECM constitutes the non-cellular component of tissues and is composed of structural and functional proteins. Each tissue has a unique ECM signature that provides instructional cues for cellular differentiation. Therefore, tissue engineering strategies have focused on mimicking the composition of the ECM to facilitate regeneration of tissues such as bone, cartilage and dentin. The importance of ECM in regulating cell behaviour is now well established and this concept is critically important for stem cells. The instructive cue of ECM on stem cells is not only dependent on the biochemical activity but also on the biophysical and biomechanical properties.

METHODS: In the present study we have developed biomimetic ECM based scaffolds consisting of cell secreted ECM incorporated within a copolymer matrix of collagen and chitosan for hard tissue engineering. In order to regenerate hard tissues, we generated native ECM of differentiating human marrow stromal cells (HMSCs) to facilitate osteoblast differentiation. Scanning electron microscopy, Magnetic resonance imaging (MRI) and Nano-indentation techniques were used to characterize the surface and mechanical properties of the ECM embedded scaffolds and the control collagen-chitosan scaffold. The potential of mesenchymal stem cells (MSCs) to differentiate into osteogenic cells when seeded within the biomimetic ECM scaffold were ascertained by RT-PCR. In-vivo implantation studies were performed to assess the osteogenic potential of the ECM based scaffold. In-vitro nucleation experiments were performed to determine the mineralization potential of the scaffold.

RESULTS: For bone tissue engineering, differentiating HMSCs were used for synthesizing the ECM scaffold. These cells secreted a cell instructive structural ECM containing a repository for growth factors, matrix proteins etc. all within a 3-dimensional organization when cultured in osteogenic medium for several days. culture medium. Real-time PCR data showed that the ECM incorporated scaffold had the potential to induce differentiation of mesenchymal stem cells into osteoblasts without the need for external addition of growth factors or differentiating agents. MRI analysis showed that the ECM scaffold seeded with HMSCs showed a reduction in relaxation time indicating osteogenic differentiation and matrix mineralization resulting in increased stiffness of the matrix. The diffusion characteristics showed that the osteogenic scaffold allowed diffusion of nutrients. In vitro nucleation studies show that the ECM scaffold favored the nucleation of hydroxyapatite both under physiological and high concentrations of calcium and phosphorus ions.

DISCUSSION & CONCLUSIONS: Biomaterials can be designed to interact with cells utilizing biomimetic principles. The ECM scaffold in this study served to provide the mechanical environment and microenvironment for cells. The structural and mechanical properties of the scaffold permitted cell survival and proliferation. Differentiation of stem cells to osteogenic lineage was facilitated by the embedded information in the ECM and specialized receptors on the surface of HMSCs transmitted this information as biochemical signals which facilitated the osteogenic differentiation of HMSCs.

The ideal scaffold for bone tissue engineering should have the ability to nucleate calcium phosphate polymorphs. The presence of matrix molecules within the ECM scaffold permitted the nucleation and growth of calcium phosphate polymorphs. Developing such ECM based biomaterials will eliminate the need for growth factor delivery in tissue engineering applications. This scaffold has the potential to replace the traditional triad of cells, scaffolds and growth factors with a duo of just cells and scaffolds.

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Biocompatibility problems: an alternative method to approach *in vitro* testing?

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INTRODUCTION: Biomaterials are designed to improve tissue regeneration. *In vitro* testing is a helpful tool to determine the behavior of the biomaterial *in vivo*; however, there are some limitations and often, *in vitro* results do not correspond to the *in vivo* outcome[1]. The inflammatory and immune response following implantation is something to be accounted for[2], and it cannot be analyzed in *in vitro* experiments only using osteoblastic cell lines [3]. Connective tissue formation is a handicap for good osteointegration in dental implants. Inflammation is mediated by macrophages, and these can occur in two different states, pro-inflammatory M1, and anti-inflammatory M2[4]. The first ones are characterized by secreting more pro-inflammatory cytokines. Herein, we propose an alternative and parallel *in vitro* testing, using the RT-PCR method to determine cell differentiation of osteoblasts and macrophages polarization state, when the cells are grown on different biomaterial surfaces.

METHODS: The osteoblast cell line MC3T3-E1 and the macrophage cell line RAW 264.7 were both grown on titanium discs coated with the different biomaterials derived from sol-gel formulations (REF) at 37ºC with 95% humidity. MC3T3-E1 osteosarcoma was used to test cytotoxicity, ALP activity and cellular proliferation, as well as gene expression of osteogenic markers (OCN, OPN, COL I) with RT-PCR. Macrophage phenotype was assessed pro- and anti-inflammatory gene expression on macrophages using distinctcytokines.

RESULTS: Osteogenic genes expression by RT-PCR grown on polystyrene plates at 14 and 21 days (Fig.1).

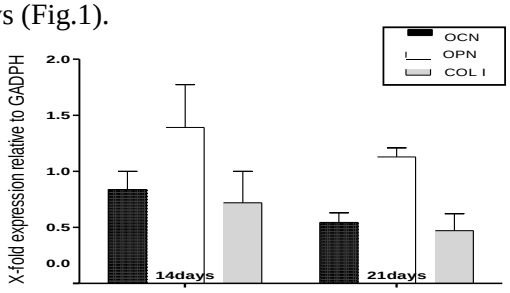

Fig.1: Osteogenic gene expression at 14 and 21 days

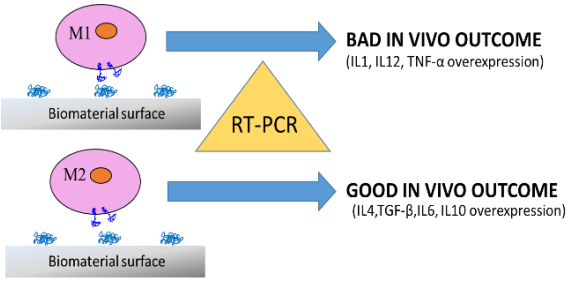

Fig. 2: Detection of macrophages polarization when grown on different biomaterials surfaces.

DISCUSSION & CONCLUSIONS: Previous studies in our group, suggest that proteins adhered to the biomaterial surface can correlate with the in vivo outcome of the implant. Therefore, we propose that the analysis of these proteins could predict materials biocompatibility. Ultimately the microenvironment between the cell and the material surface will be defined by the proteins adhered to the implant surface, that will activate a natural immune response *in vivo*. The method we propose (Fig.2) is based on the characterization of macrophages phenotype, that may have a more defining role on addressing implant biocompatibility than the classic osteoblast testing, depending on the phenotype activated by the first layer of proteins adhered to the biomaterial surface.

ACKNOWLEDGEMENTS: This work was supported by the MAT 2014-51918-C2-2-R (MINECO), P11B2014-19, Plan de Promoción de la Investigación de la Universidad Jaume I (Predoc/2014/25), Generalitat Valenciana (Grisolia/2014/016) and Ilerimplant S.L.

Synthetic intrinsically-disordered-peptides for bone tissue engineering

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INTRODUCTION: Vertebrate biomineralisation is closely associated with intrinsically disordered proteins (IDPs). Proteins closely related to hydroxyapatite crystal formation, growth control and orientation contain intrinsically disordered regions. Furthermore IDPs are involved not only in biomineralisation, but are also involved in inflammatory responses in bone and teeth [1]. The proline-rich regions of these proteins have remained remarkably well-conserved through evolution, suggesting that they may have functional importance. In previous studies, artificial consensus peptides based on these conserved domains were synthesized. The designed peptide contains a polyproline sequence of 25AA length, further comprising a systematic variation of non-proline residues [2,3]. We hypothesized that IDPs could influence biomineralization and promote bone regeneration.

METHODS: Human primary osteoblasts (hOBs) were seeded in cell culture plates. When confluent, hOBs growth media was supplemented with the different IDPs (Table 1) at a final concentration of 2.5 μM. As positive control, Emdogain™ (EMD), a purified extract of proteins from enamel matrix, extracted from porcine developing teeth, mainly constituted by amelogenins, and clinically used for bone regeneration, was added to growth media. As well, cells without supplementation were used as negative control (C). Cytotoxicity of the IDPs was evaluated after 1 and 3 days. Gene expression of osteogenic markers was evaluated after 1, 3, 7 and 14 d using qPCR. Alkaline phosphatase (ALP) activity and calcium deposition were analysed after 21 d. Additionally, a wound healing *in vitro* assay was performed. Minimum two out of four hOBs donors (3-4 replicates each) were used for the experiments.

Table 1. Aminoacid (one-letter code) sequence of the IDPs used.

Name	Sequence
P2	PLVPSQPLVPSQPLVPSQPQPPLPP
P5	PLVPSSPLVPCCPLVPCCPSPPLPP
P6	PHQPMQPQPPVHPMQPLPPQPPLPP

RESULTS: As expected, no cytotoxic effect of the IDPs was observed. Cells treated with P2, upregulated the expression of the osteogenic markers collagen-Iα1 and alkaline phosphatase at earlier time points than control- and EMD-supplemented groups. Both, cells treated with P2 and EMD, increased calcium deposition although P2 effect was not statistically significant. EMD osteogenic effect did not involve ALP expression or activity upregulation. Cells treated with P2 showed the smallest open wound area after 24 h.

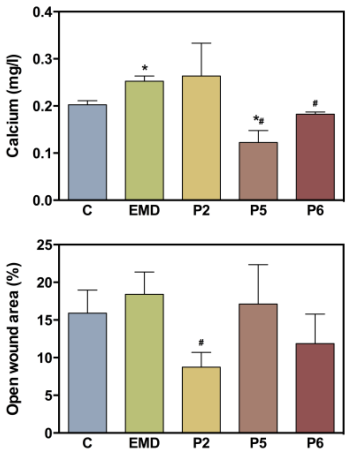

Fig. 1: (Up) Calcium deposition on hOBs extracellular matrix cultured for 21 d with the different IDPs or controls. (Down) Open wound area after 24 h of in vitro healing in presence of different IDPs or controls. P≤ 0.05: (*) vs. C; (#) vs EMD.

DISCUSSION & CONCLUSIONS: Results show that synthetic IDPs are able to influence hOBs biomineralization. The earlier upregulation of osteogenic markers together with the decreased wound area induced by P2 indicate that it has potential to induce bone regeneration. Bone-related proteins, cytokines and chemokines released to culture media are already being analysed to confirm these results. IDPs might be a reliable option to develop new biomaterials and tissue engineering strategies.

ACKNOWLEDGEMENTS: This study was supported by the NFR 231530.

Porous bioactive sol-gel inorganic/gelatin class II fibres via solution blow spinning

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INTRODUCTION: Recently solution blow spinning (SBS) has been used to make bioactive nanocomposites fibres [1], sol-gel fibres, as well as porous inorganic/gelatin hybrids via cryogenic SBS [2]. In this work formulations based on thermoreversible precursors with tuneable viscosities are developed, taking advantage of the thermally activated chain confirmation change of gelatin. Gelatin is functionalised with with (3-glycidyloxypropyl)trimethoxysilane (GPTMS) to produce class II hybrid fibres, which exhibit covalent cross-links between organic and inorganic phases and hence control physical properties, dissolution and degradation. The challenge of introducing calcium into the inorganic network is also addressed. The *in vitro* degradation and bioactivity of calcium containing fibres was investigated by a 21-day incubation period in simulated body fluids (SBF) as well as cell culture with osteoblast-like cells for the same period. Non-calcium containing hybrid fibres were used as controls.

METHODS: Gelatin derived from porcine skin (Type A, gel strength ~300 g bloom) was dissolved at 10% w/v in Dulbecco's phosphate buffered saline (PBS, pH 7.4), and functionalised with GPTMS (92 $\mu L \cdot g^{-1}$), using a method adapted from [3]. The sol-gel precursor was prepared as a separate solution compromising of tetraethyl orthosilicate (TEOS), *tert*-butyl alcohol (TBA), water and 1M HCl (acid catalyst for hydrolysis) added in that order to give a molar ratio of 1:5:2.7:0.05 (TEOS:TBA:H_2O:HCl). The sol was then roller mixed for 24h. Separately, calcium methoxide ($Ca(OCH_3)_2$, SigmaAldrich) and calcium ethoxide ($Ca(OEt)_2$ lab made) were incorporated into the inorganic formulations to give a molar ratio of 0.7:0.3:5:2.7:0.05 (TEOS: $Ca(OCH_3)_2$ or $Ca(OEt)_2$:TBA:H_2O:HCl). Ethylene glycol was used to assist the solubility of $Ca(OCH_3)_2$. Any $Ca(OEt)_2$ that did not solubilise was centrifuged then the supernatant was mixed with the organic precursor. The class II, class II-$Ca(OCH_3)_2$, and class II-$Ca(OEt)_2$ precursor solutions were then spun into fibres using cryogenic SBS [2].

RESULTS: Class II-$Ca(OEt)_2$ had a yellowish colour indicating that $Ca(OEt)_2$ was present and the precursor solution exhibited thermoreversible properties similar to the class II control precursor. The class II-$Ca(OCH_3)_2$ precursor rapidly gelled due to the presence of $Ca(OCH_3)_2$ giving a narrow window for processing into fibres. Scanning electron microscopy (SEM) revealed that both class II and class II-$Ca(OEt)_2$ had a rough surface topography. X-Ray diffraction (XRD) confirmed the formation of hydroxyapatite in the calcium containing fibres.

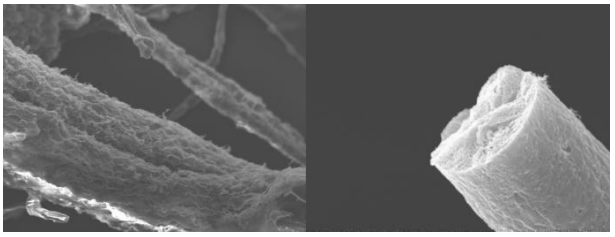

Fig. 1: Images of Right: class II-$Ca(OEt)_2$ fibres, and Left: class II fibre. Fibre diameters within range 700 nm – 5 μm.

DISCUSSION & CONCLUSIONS: Calcium containing sol-gel inorganic/gelatin class II fibres were successfully made. However, it remains a challenge to incorporate calcium using alkoxide precursors at the desired molar concentrations due to their rapid polycondensation and their poor solubility.

ACKNOWLEDGEMENTS: The experimental assistance of Babak Jazayeri is appreciated.

Porcine mandible blocks as a novel method for assessing biofilm formation and removal in class two furcation defects *in vitro*

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INTRODUCTION: Periodontal disease is a common pathological condition of the oral cavity that can ultimately lead to tooth loss.[1] Bone loss around multi-rooted teeth, such as class two furcation defects are frequent [2] and considered hard to treat.[3] Long term outcome of periodontal disease largely depend on successful removal of biofilm. This can however also modulate the root surface. The aim of the present study is to develop an in vitro model for the assessment of biofilm formation and removal in periodontal class two furcation defects in the porcine mandible. Micromechanical effects of the treatment are also included in the study outcome.

METHODS: Blocks of porcine mandibles with molar 3 were prepared (n=100). Buccal class two furcation defects were created using a Rosen burr. Samples then autoclaved, dried and covered with silicone leaving only the furcation area exposed. Furcation was inoculated with bioluminescent *S. epidermidis* Xen43and incubated for 24 hours to form a biofilm. Samples were randomly assigned to treatment groups and biofilm removed by curette (Gracey 1-2), ultrasonic tip or Waerhaug diamond. Each treatment was performed for 30 seconds. Following the treatment, the silicone was removed and the teeth separated from the bone. Only the teeth were used for analyzes. Positive control with no treatment was used as baseline. Remaining biofilm was detached from the root surface by bath sonication and dilution series was prepared (up to 10^{-6}). Diluted samples were plated on tryptic soy agar plates and CFUs were counted after 24 h incubation. 15 µl of 10^{-3} dilution of each sample was resuspended in 185 µl of tryptic soy broth and incubated in a multi-purpose plate reader for 18 h while luminescence was measured every 15 min during the entire incubation period. Micromechanical damage caused by the treatment was assessed by SEM imaging.

RESULTS: SEM scan shows a successful seal using the silicone with bacterial biofilm contained within the area of interest (Fig1). Micromechanical damage was clearly visible in all treatment groups when assessed in SEM. Initial results from the plated samples indicate that bacterial biofilm is slightly reduced using all three methods of debridement compared to positive control.

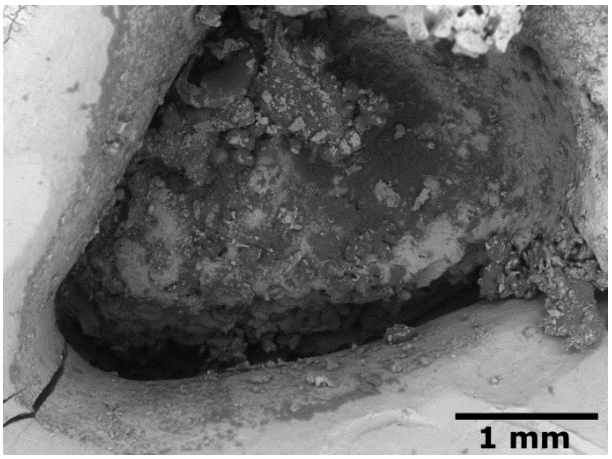

Fig 1: Biofilm in class two furcation.

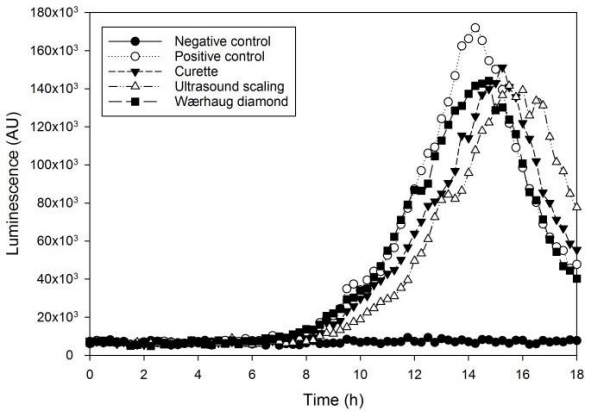

Fig 2: Luminescence of bacteria remaining on the root surface after treatment.

DISCUSSION & CONCLUSIONS: The model presents a novel method for assessing biofilm and micromechanical damage following debridement in class two furcation defects. Results indicate that the model offer a reliable method for evaluating the biofilm formation and subsequent removal from the furcation area. SEM scanning consistently show the mechanical damage asserted by debridement tools.

Role of fibrinogen conformation for the adhesion of *S.epidermidis* on surface immobilized SiO_2 nanoparticles

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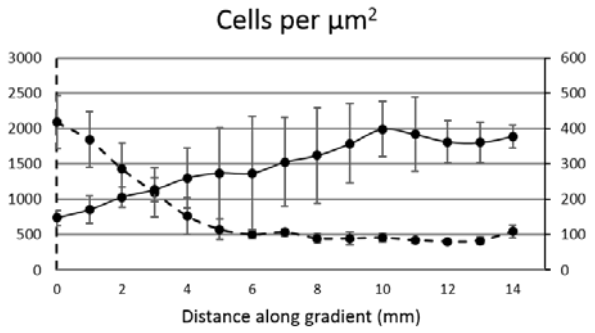
INTRODUCTION: Bacterial cells have been shown to respond to nano-sized surface features and these may therefore be utilized as a strategy for reducing the adhesion and survival of bacteria on biomaterial surfaces. However, the underlying mechanism(s) for bacterial adhesion on nanostructured materials is still largely unknown. Here we have used gradients in surface nanotopography in a laminar flow chamber to study how the total cell-surface contact area affects the initial adhesion of *S.epidermidis* to nanostructured surfaces. Additionally, circular dichroism (CD) was used to assess changes in the secondary structure of pre-adsorbed fibrinogen upon adsorption to the surfaces.

METHODS: Gradients with 40nm sized SiO_2 nanoparticles were prepared by binding the particles to a SAM of APTES (aminopropyl triethoxysliane) on standard microscope glass slides using a diffusion method to arrange the particles with increasing distance (up to ~100nm) along the surface. The space in between the particles was then functionalized with NHS-PEG (5 kDa) to create a gradient of "islands" of SiO_2 particles against a non-adhesive PEG background. Additionally, some surfaces were heat treated for 60min at 400 °C to achieve uniform surface chemistry (hydrophilic) and pre-incubated with human fibrinogen before bacteria was incubated on the surfaces in the flow chamber.

S. epidermidis (O.D 1.5) was incubated at room temperature in PBS buffer on the gradient nanostructured surfaces mounted in a parallel flow chamber for durations ranging from 15s to 10min, and then subjected to laminar flow shear stress between 80-600 Dynes. Cells were stained with acridine orange and images were taken every mm along the gradient using fluorescence microscopy. Cells were then counted using the software ImageJ. Samples were also viewed in SEM tilted at an angle of 60° to study the interface between the cell and substrate.

For CD measurements of protein secondary structure, 10 quartz glasses (thickness 0.1mm) with or without immobilized SiO_2 nanoparticles were stacked with 0.5mm spacing in a custom made holder and immersed in fibrinogen solution (1mg/ml) in PBS buffer for 60min and thereafter rinsed with copious amounts of PBS before measurements.

RESULTS: After incubation for 10min and subjected to a shear stress of 400 dynes for 5min the number of adhered bacteria on PEG-ylated gradients correlated with the number of available surface area (number of particles per μm^2). Intriguingly, when the gradient instead was pre-adsorbed with fibrinogen an inverse correlation was found (see figure below). Results from circular dichroism measurements showed a more native-like secondary structure of fibrinogen adsorbed on nanostructured than on smooth substrates when compared to a bulk solution of fibrinogen.



DISCUSSION & CONCLUSIONS: We have shown that the number and spacing between SiO_2 nanoparticles is crucial for the attachment of *S.epidermidis*. In addition, the secondary structure of fibrinogen retains a more native conformation when adsorbed onto immobilized 40nm SiO_2 particles than to a smooth surface with the same surface chemistry. We also show that the native conformation of fibrinogen attenuates adhesion of *S. epidermidis* although the cell-surface contact is larger.

Effect of irradiated TiO_2/H_2O_2 suspensions on *Staphylococcus epidermidis* biofilm

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INTRODUCTION: Peri-implantitis is an inflammatory disease associated with dental implants defined as bleeding by probing and marginal bone loss [1]. A number of treatment methods exist today, but none are satisfactory. Titanium dioxide has three common crystalline forms - anatase, rutile and brookite. By irradiating anatase with light having $\lambda < 384$ nm or rutile with $\lambda < 410$ nm [2], valance electrons can be excited to the conduction band and reactive oxygen species (ROS) may be created when excited electrons and holes react with oxygen or water. These ROS can efficiently inactivate bacteria by damaging (oxidizing) the cell wall of the bacteria. The aim of this study was to determine which crystalline structure, concentration of hydrogen peroxide (H_2O_2) and wavelength of light irradiation most efficiently inactivates a *S. epidermidis* biofilm.

METHODS: Precultured *S. epidermidis* were mixed with Tryptic Soy Broth (TSB) and inoculated for 24h at 37 °C. OD was adjusted to 0.02 ($\approx 2 \cdot 10^7$ colony forming units (CFU)/mL). Subsequently 550 µl of the suspension was added to 48 well plates containing sandblasted and acid etched titanium grade 2 coins and inoculated for 8 h at 37 °C to culture a biofilm. The coins were then soaked in a suspension of different combinations of H_2O_2 and 0.5 g/l of anatase/rutile (A/R) or rutile particles and irradiated with light having $\lambda = 365$ or 405 nm, or ambient light. The intensities of the light was 2.5 or 17 mW/cm^2 with $\lambda = 365$ or 405 nm. Coins were then immersed in fresh TSB and luminescence was measured every hour in a Hidex Chameleon microplate reader as a means of determining bacterial viability.

RESULTS: As seen in Fig. 1a, rutile is more effective than A/R with $\lambda = 405$ nm in inhibiting biofilm regrowth. Fig. 1b and c show that an increased biofilm inhibition was obtained for test groups with 6 % H_2O_2. Fig 1d shows that irradiation of light with $\lambda = 365$ nm was more effective than 405 nm. The effect of H_2O_2 tends to decrease when the concentration is decreased to 3%. High intensity light (17 mW/cm^2), is not significantly better than ambient light (figures not shown.)

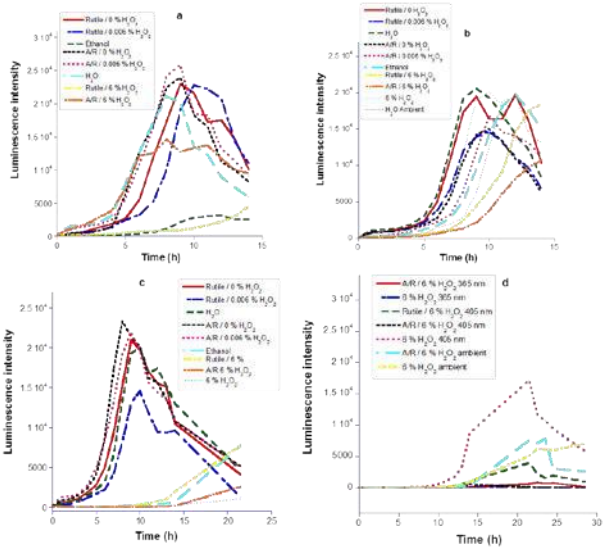

Fig 1. a) Luminescence intensity measurements of titanium coins exposed to light with $\lambda = 405$ nm for 1 min. b) 365 nm for 1 min. c) Ambient light for 1 min. d) 365 nm, 405 nm and ambient light for 1 min.

DISCUSSION & CONCLUSIONS: H_2O_2 had the largest effect on bacterial viability of the biofilm with increased effect at higher concentration. The presence of TiO_2 particles also increased the effect slightly. Light irradiation at $\lambda = 365$ nm provided increased effect, but only marginal effect at $\lambda = 405$ nm.

ACKNOWLEDGEMENTS: This research was funded by Vinnova through the Eurostar program.

Silk-assembly enables integration of viable cells into engineered tissue

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INTRODUCTION: Tissues are built from cells integrated in an extracellular matrix (ECM), mainly composed of a three-dimensional (3D) protein fiber network with specific sites for cell anchorage. By genetic engineering, motifs from the ECM protein fibronectin can be functionally fused to a recombinant silk protein, FN-silk, with propensity to self-assemble into various fibrillar formats under physiological-like conditions[1]. Herein we show that dispersed mammalian cells can be added to the FN-silk protein solution before assembly, and thereby together form 3D tissue-like constructs (Figure 1).

METHODS: A collection of various adherent mammalian cell types were integrated into FN-silk in the format of fibre and foam. Cells within the silk scaffolds were analysed using Alamar Blue and Live/dead viability assays and subjected to various immunostainings both directly in scaffolds and after cryosectioning. Uni-axial tensile testing was performed under physiological conditions to determine the mechanical properties of fibres with viable cells.

RESULTS: The proliferative capacity of the cells within the FN-silk scaffolds was maintained through more than two weeks, with 82-98% viability for all cell types investigated. Presence of proliferative cells within the deepest parts of the materials was confirmed by staining of cryosections.

Scaffold-connected cells with pronounced spreading and well-organized actin stress fibres were found within both foam and fibres. Elongated residuals of cells with teared off cytoskeleton were found after the fibres had been stretched to over twice of their initial length, indicating a force transition into and throughout the fibre.

Tissue-like 3D constructs with vessel formations could be obtained by addition of a small fraction (3-10%) of endothelial cells during assembly.

CONCLUSIONS: Herein we present a novel route for "bottom-up" manufacturing of tissue-like constructs with viably integrated cells, and at the same time sufficient mechanical flexibility. A recombinantly produced silk protein, genetically decorated with components from the extracellular matrix, is allowed to assemble under physiological conditions in presence of selected cells. This result in the formation of macroscopic constructs containing well-spread, scaffold-connected and proliferative cells with tissue-like morphology, including formation of vasculature.

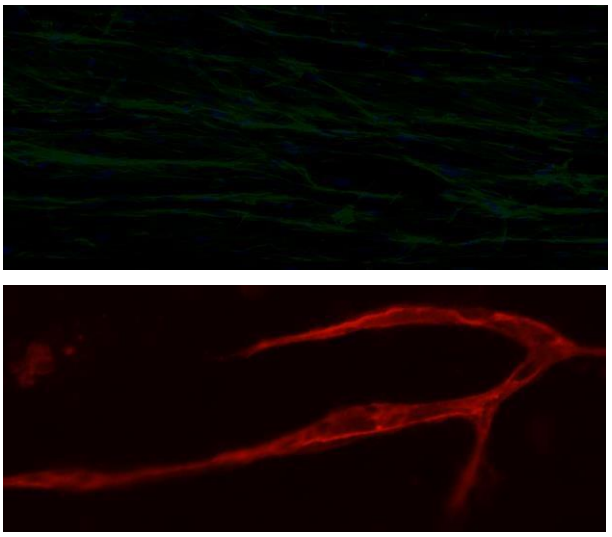

Fig. 1: Upper: F-actin and Dapi staining of a FN-silk fibre with integrated human primary fibroblasts. Lower: Micrograph of long and branched vessel-like structures in FN-silk foam after culture for 10 days with integrated human mesenchymal stem cells and endothelial cells.

ACKNOWLEDGEMENTS:

Spiber Technologies AB is acknowledged for proving silk proteins.

Biocompatibility and mesenchymal stem cells adhesion on additively manufactured and magnetically assisted finished 316L stainless steel

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INTRODUCTION: Orthopaedic implant-technology nowadays, has come a long way in enhancing patient's life after surgery but there still are some issues regarding the osteointegration of implants, leading to infections and experienced pain. Additive manufacturing (AM) creates the basis for this research, being able to produce highly customizable implants. Lawrence E. Murr describes the forefront of additive manufacturing and its potential uses in orthopedy in his article from 2012 [1]. Indicating the positive factors of bone ingrowth on 3D AM scaffolds using cells of the osteoblastic lineage. To our knowledge however, there aren't any studies looking at MSC adhesion and proliferation, on AM 316L steel. This will be important to study, in order to gain insight on AM orthopaedic applications, as the first cells to be recruited at the implant site are the MSCs [2]. Hence, the scope of this research is to study AM 316L stainless steel samples, finished by magnetic assisted finishing (MAF) and look at the effects of the surface topography on the adhesion of MSCs.

METHODS: Samples are additively manufactured using M2-Concept based on powder-bed fusion technology and 316L metallic powder and subsequently surface finished using MAF. Biocompatibility of the metallic material is being assessed using enzyme-linked immunosorbent assay (ELISA) and Limulus amoebocyte lysate (LAL) assay. Mesenchymal stem cells (MSC) are then cultured on the samples for 48h, fixated and subsequently marked using Phalloidin 488 and Draq5 633. Confocal microscopy is then used to assess MSC adhesion.

RESULTS: ELISA and LAL tests both resulted clean and the first MSC adhesion test-results show good adhesion-conditions for surfaces roughness's in the low micrometre range. The control samples, which are not subject to any surface finishing, show low adhesion characteristics, thus suggesting the beneficial impact of the MAF-finishing process on the samples.

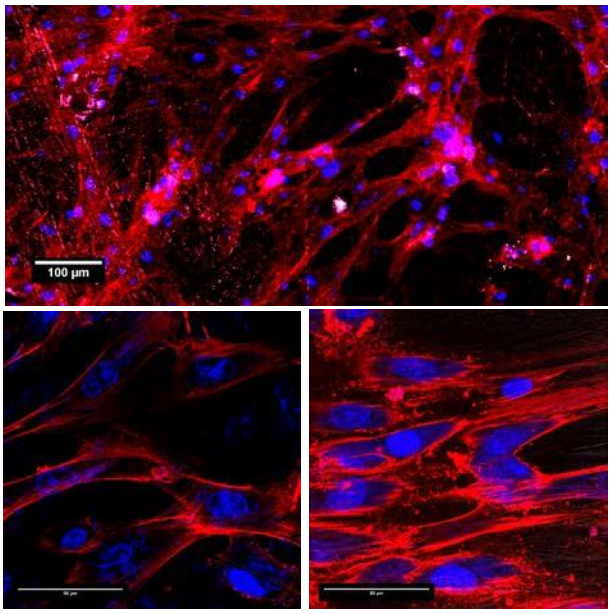

Figure 1: Top shows a 10X magnification of adhered MSC onto a sample with a surface roughness of 0.62µm. Bottom left and right images show a 63X magnification of two separate samples with roughness's corresponding to 0.25µm and 0.62µm respectively. Scale bars on bottom images are 50µm.

DISCUSSION & CONCLUSIONS: Preliminary results suggests the beneficial impact of MAF on the test-samples. The 3D-printing process does not seem to have an impact on the toxicity of the metal, as both ELISA and LAL tests resulted clean and given the cell survival during performed tests.

Collagen-hyaluronan in colloidal silver

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INTRODUCTION: Collagen and hyaluronan are natural biopolymers occurring in the human body as a materials for scaffold in tissue engineering, as a components of a bandage by wound healing processes, as an alternate fluid in human joints, as a space filling matter in plastic surgery and last but not least as a hydration matter in cosmetics. Hyaluronan exists in high concentrations during fetal skin development, is involved in cell migration and differentiation, and is the first macromolecule to appear in the ECM during tissue engineering repair.[1] These two biomaterials dissolve in colloidal silver have been studied in this work for biomedical applications.

METHODS: Hayluronan HyActive (13kDa) from Contipro Biotech (Czech Republic) is produced biotechnologically and extracted from the cell walls of the bacteria *Streptococcus zooepidemicus*. Collagen from Inventia Polish Technologies Sp. z o.o., obtained from fish skins. Colloidal silver (20 ppm) obtained from Antibakterin (Czech Republic). The physical properties and finding of suitable concentration of biomaterials were measured by three methods. The suitable concentration and ratio of collagen-hyaluronan were measured by High resolution ultrasonic spectroscopy from Ultrasonic Scientific (Ireland) [2] and Densitometer DSA 5000M from Anton Paar (Austria). The rheological properties were measured by Discovery Hybrid Rheometer from TA Instruments (USA).

RESULTS: By densitometric measurement was found that collagen-hyaluronan in colloidal silver was stable in temperature 20-50°C.
Collagen-hyaluronan colloidal silver spray was tested for healing on human skin (figure 1), for burn, frostbite, bedsore, varicose ulcer etc. Due to colloidal silver this spray could be applicate to raw wound.

CONCLUSIONS: The combination of collagen, hyaluronan and colloidal silver in ratio collagen:hyaluronan, 1:1 is the best ratio for good application in a spray form for healing of wound.

Fig. 1: Healing after application of collagen-hyaluronan spray.

ACKNOWLEDGEMENTS: This research was supported by project No. LO1211 from the Czech Ministry of Education.

Effect of cationic doping on grain boundary corrosion in TiO_2 bone scaffolds

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INTRODUCTION: Bone has the potential for self-regeneration[1], but needs a support structure for regeneration of critical bone defects. Ceramic scaffolds have shown excellent properties as support structure due to biocompatibility and high porosity combined with high compressive strength[2]. To improve these characteristics, the objective of this study was to investigate the influence of different cations on TiO2 slurries and furthermore the architecture, mechanical properties and grain boundary corrosion of TiO2 bone scaffolds.

METHODS: The influence of K^+, Na^+, Ca^{2+}, Sr^{2+}, Mg^{2+} cations on aqueous TiO2 slurries and the microstructure of ceramic bone scaffolds was investigated. The scaffolds were prepared by using the foam replication method and the chloride salts of the cations were added into acidic (pH 1.6-1.7) aqueous anatase dispersions. The zeta potential of dispersed TiO_2 particles in d-H_2O and 0.1M concentrated solutions and the viscosity of prepared slurries were measured. While scaffold architecture was analysed using microCT, microstructure was investigated via SEM and EDX. As mechanical property, the compressive strength was measured. After storing the scaffolds in d-H_2O and an acidic solution (pH3) for 2 and 4 weeks the compressive strength was measured again and the grain boundaries were investigated by using SEM.

RESULTS: The influence of all cations resulted in a decreased zeta potential between pH 1.6-1.7. Na^+ cations showed an inert electrolyte influence whereas divalent salts indicated non-inert influence. Thereby the viscosity of all slurries was increased, whereby the influence of divalent salts was higher. The scaffold's properties showed increased strut size and compressive strength with similar porosity which are important properties for successful osseointegration. Furthermore, Sr^{2+} and Ca^{2+} ions resulted in coarser grain size and prominent grain boundary phases. Scaffolds prepared with monovalent cations showed similar compressive strength after 4 weeks in an acidic solution while divalent cation scaffolds showed decreased compressive strength and corroded grain boundaries.

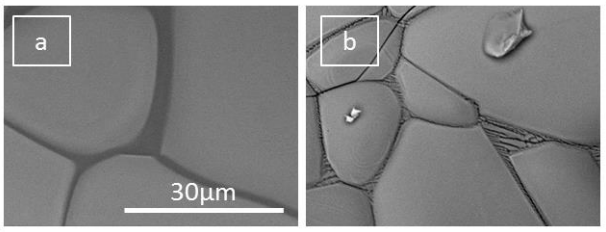

Fig. 1: SEM images of 0.1M $CaCl_2$ scaffold grain boundaries after 2 weeks in dH_2O (a) and pH3 (b).

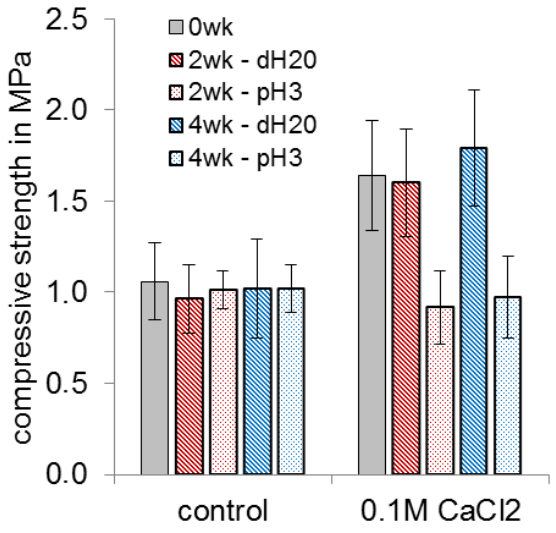

Fig. 2: Compressive strength of control and 0.1M $CaCl_2$ scaffolds at 0, 2 and 4 weeks.

DISCUSSION & CONCLUSIONS: As a result adding divalent cations in TiO2 slurries caused higher viscosity, strut size and compressive strength without reducing porosity, but decreased compressive strength after 2 weeks in acidic solution. Further investigations of grain boundary composition in Ca^{2+} and Sr^{2+} scaffolds are required.

ACKNOWLEDGEMENTS: This work was supported by the Research Council of Norway grant no. 228415.

Composite alginate/hydroxyapatite containing selenium and risedronate

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INTRODUCTION: The main goal of this work is to develop and characterize a multifunctional composite bone substitute preventing complications of bone metastasis. The project involves the synthesis of nanocrystalline hydroxyapatite enriched in selenium (IV) oxyanions. Selenium is a trace element of human tissues, having a great impact on bone density and bone metabolism. It has been reported that selenium may be a chemopreventive agent in various types of cancers and metastasis [1,2]. Risedronate sodium is a bisphosphonate using as antiresorptive drug for the treatment of osteoporosis and bone tumours [3].

METHODS: Hydroxyapatite containing SeO_3^{2-} ions (SeHA) was synthesized according to the wet method described in [4]. Nanocrystalline SeHA was physicochemically studied using FT-IR, PXRD, WD-XRF and ssNMR methods. SeHA powder was used to prepare porous microgranules. A viscous aqueous solution of alginate sodium (ALG) was mixed with fine SeHA powder, NH_4HCO_3 and risedronate sodium (RIS). Then, the obtained slurry was used to produce the microgranules (in $CaCl_2$ solution). The obtained microgranules were washed with distilled water and dried at 40˚C for 48 h. The RIS/ALG/SeHA materials were analysed using FT-IR, SEM and ssNMR methods. For comparison, the microgranules without risedronate sodium were also produced and studied.

RESULTS: The physicochemical analysis (PXRD, FT-IR, WD-XRF) of the SeHA sample have shown that it is nanocrystalline hydroxyapatite containing ca. 9.6 wt% of selenium in selenite form (Table 1). The RIS/ALG/SeHA microgranules are porous (SEM). Risedronate interacts with calcium cations from SeHA sample (^{31}P and ^{13}C NMR). Risedronate adsorbs on the SeHA nanocrystals..

DISCUSSION & CONCLUSIONS: In this project we have successfully produced microgranules containing nanocrystalline hydroxyapatite enriched in selenium and risedronate. Our future work will focus on RIS and Se release studies and in vitro tests.

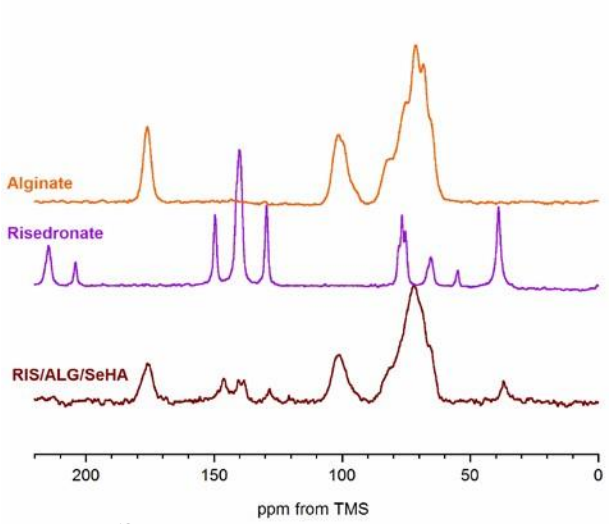

Fig. 1: ^{13}C CP MAS ssNMR of the obtained microgranules and their components: alginate and risedronate.

Table 1. Main characteristics of the SeHA sample.

	Crystallite size (nm)	Se content (wt. %)
SeHA	10±2	9.6

ACKNOWLEDGEMENTS: This work was supported by the research program (Project NCN-2011/03/D/ST5/05793) of the Polish Ministry of Science and Higher Education.

Metal oxides, hydroxyapatite and bone healing

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INTRODUCTION: Magnesium and its alloys have been suggested [1, 2] for application as biodegradable metal implants, but there is still a lack of understanding of the biological response to its corrosion products and their influence on the local tissue response at the implantation site. The approach in this study is to implant MgO in rat tibia and follow the healing process during 1-3 weeks of healing comparing the results to those of sham-operated controls.

METHODS: The healing bone was analysed by histology, imaging mass spectrometry (ToF-SIMS) and SEM equipped with an EDX-analyser, as described previously [3, 4].

RESULTS: The normal healing process, comprising callus bone after 1 week of healing, followed by remodeling and completed healing after 3 weeks was little affected by MgO when examined by histology. However, the EDX-analysis showed a high content of mineral in the bone marrow after 1 week of healing with implanted MgO.

In-vitro studies of MgO incubated in tissue culture medium confirmed that MgO catalyses the formation of calcium phosphate, shown by ToF-SIMS to be hydroxyapatite (HA). The HA-coated MgO was then incubated with cultures of embryonic stem cells. The stem cells produced mineral after exposure to HA-coated MgO, as shown by von Kossa staining. Thus, we have defined two different sources of MgO-induced bone mineral that may be active during bone healing in vivo.

In a third set of experiments, we used other metal oxides that have been reported to stimulate bone formation, MnO and ZnO. The MnO and ZnO where more efficient than MgO in catalyzing the formation of hydroxyapatite, and were also more efficient in promoting bone healing in vivo.

DISCUSSION & CONCLUSIONS: The results of the present study suggest that the formation of hydroxyapatite is induced in the bone marrow after implantation of MgO, MnO or ZnO. Two sources of the apatite were defined, a direct catalytic effect of the oxides in contact with tissue fluid and an activation of human embryonic stem cells that are exposed to the HA-coated metal oxides.

The catalysis of HA-formation by magnesium corrosion products and the production of HA by cells has been shown previously [5, 6]. The generalization of the phenomenon to ZnO and MnO is a novel finding. The catalysis of HA by metal oxides is also seen with TiO2 in contact with tissue fluid [4].

ACKNOWLEDGEMENTS: This project has been generously supported by Elos Medtech AB, Sweden.

Knitted 3D scaffolds of polybutylene succinate support human adipose stem cell attachment, proliferation and osteogenic differentiation

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INTRODUCTION: Polybutylene succinate (PBS) is an aliphatic biodegradable polyester with different processability and mechanical properties compared to polylactides (PLA)s, which are the most commonly used synthetic polymers in tissue engineering (TE). Currently only few studies have evaluated PBS-containing materials for bone TE.

METHODS: Polymers and polymer blends used in this study are listed in Table 1. In addition to PLA, PBS and PLA-PBS blends, PLA-polycaprolactone (PCL) and PLA-poly(trimethylene carbonate) (PTMC) blends were tested as reference materials. Polymer fibers were prepared using the melt spinning procedure. Knitted and rolled scaffolds (height 5 mm, diameter 10 mm) were manufactured, seeded with human adipose stem cells (hASCs) and cultured for up to 27 days in osteogenic medium. Cell spreading inside the scaffolds was evaluated by microcomputed tomography. Moreover, hASC viability, attachment and proliferation in the scaffolds were determined. The osteogenic differentiation was evaluated with alkaline phosphatase (ALP) activity and mineralization assays.

Table 1. Polymer proportions of the blends used in the scaffold manufacturing.

Material	PLA (weight-%)	Other component (weight-%)
PLA	100	0
PBS	0	100
25% PBS	75	25
5% PBS	95	5
5% PCL	95	5
5% PTMC	95	5

RESULTS: All the scaffolds had open pores and porosity of 67.5±6.0 %, and cells were distributed evenly throughout the scaffolds. All the scaffolds supported hASC viability but cell spreading along the fibers was only detected in PBS-containing scaffolds (Fig. 1). They also induced the strongest proliferative response and osteogenic differentiation. This was most evident with pure PBS and diminished with decreasing PBS content. On PLA, PLA-PCL and PLA-PTMC the osteogenesis was negligible.

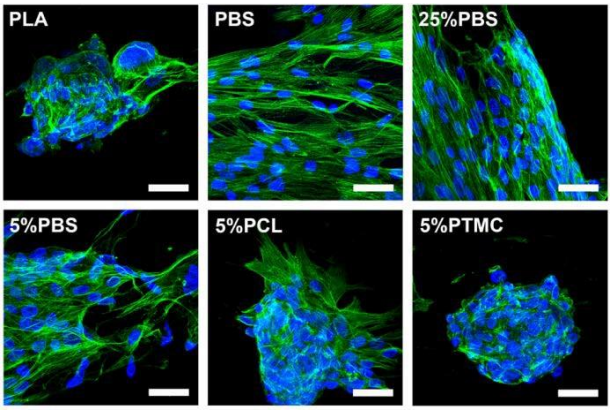

Fig. 1: Cell attachment on knitted 3D scaffolds. Phalloidin staining of the actin cytoskeleton (green) at 7d. Nuclei are stained with DAPI (blue). Scale bars 50 µm.

DISCUSSION & CONCLUSIONS: Based on our results, PBS is superior to PLA with respect to hASC attachment, proliferation and osteogenesis. This encourages to utilize PBS-based biomaterials more widely in bone TE applications.

ACKNOWLEDGEMENTS: The authors thank Sari Kalliokoski, Miia Juntunen, and Anna-Maija Honkala for technical assistance. The work was supported by TEKES, the Finnish Funding Agency for Innovation, Jane and Aatos Erkko Foundation and the Doctoral Programme in Biomedicine and Biotechnology, University of Tampere.

Calcium-alginate hydrogel beads as hosts for substrate mediated enzyme prodrug therapy

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INTRODUCTION: Cancers have a major impact on life expectancy, being a leading cause of mortality accounting for 8.2 million deaths worldwide in 2012 [1]. Current anti-cancer therapies often introduce high risks of severe adverse events due to systemic toxicity of highly potent chemotherapeutic agents. Drug eluting beads (DEB), currently employed clinically for transarterial chemoembolization (TACE), introduce local delivery lowering systemic toxicity of anti-cancer drugs. Yet, they offer low flexibility regarding changing the drug dose and drug type and terminating drug elution during an ongoing intervention. To meet these criteria, we introduce implantable Ca-alginate hydrogel beads as a biocatalytic platform for on-site activation of pharmacodynamically inactive prodrugs. Within the beads, prodrug-specific enzymes have been immobilized for adoption in substrate mediated enzyme prodrug therapy (SMEPT; *Fig. 1*) [2, 3].

Fig. 1: Schematic representation of SMEPT using biocatalytical hydrogel beads (BHB, blue sphere) and an enzyme-specific anti-proliferative drug (SN-38 glucuronide). Green spheres represent β-glucuronidase (Enz).

RESULTS: Ca-alginate beads were produced in sizes of approximately 300, 400, 600, 900, 1500 and 2000 µm allowing fine-tuned size-control of beads for embolization of specific blood vessels. Immobilization of enzymes within the beads improved enzymatic stability at 37 °C. Furthermore, increased drug activation releasing higher drug quantities were achieved by addition of increased prodrug concentrations or incorporating more enzymes in the beads. Sequential administration of prodrug under flow conditions demonstrated how local drug activation can be turned on and off by controlling the prodrug administration (*Fig. 2A*). *In vitro*, the enzyme-containing beads were successful in inhibiting proliferation of Hep G2 liver cancer cells using the SN-38 prodrug (*Fig. 2B*).

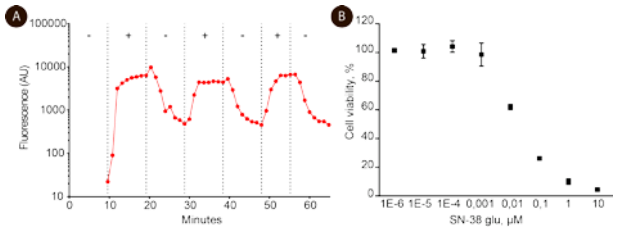

Fig. 2: (A) Sequential prodrug (non-fluorescent) administration (+/- prodrug) yields on-demand release of the corresponding "drug" (fluorescent). (B) IC_{50} on Hep G2 cells combining Ca-alginate beads containing β-glucuronidase with SN-38 glucuronide (prodrug).

DISCUSSION & CONCLUSIONS: Ca-alginate beads with embedded prodrug-specific enzymes can increase the therapeutic flexibility of DEB-like beads employed for TACE.

ACKNOWLEDGEMENTS: The European Research Council (ERC) is thanked for financial support through an ERC consolidator grant awarded to Associate Professor Dr. Alexander N. Zelikin.

Mesoporous nano–composite MCM-48/hydroxyapatite as a potential drug delivery system

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INTRODUCTION: The major aim of this project is to work out effective methods to synthesize silica/hydroxyapatite composites using ordered nanoporous silica MCM-48 and to load them with cytostatic drugs. MCM-48 is a silica material with a three-dimensional structure exhibiting la3d symmetry. Stoichiometric hydroxyapatite HA is the reference mineral in the apatite group. It is widely used in bone restorations due to its biocompatibility. In addition to the excellent biocompatibility, HA is also capable of adsorbing and releasing many molecules of great biological significance. In this project MCM-48 surface, including its internal channel walls, is being covered with HA which should improve biocompatibility of the composite and accelerate osteointegration in comparison with the two-dimensional materials.

METHODS: MCM-48 materials were prepared using hydrothermal method described by Wang et al [1]. The composite MCM-48/HA was obtained by wet method prescribed by O. A. Anunziata et al [2] for different type mesoporous silica. Obtained MCM-48 and composite MCM-48/HA were analysed by PXRD, FT-IR and TEM methods.
RESULTS: The PXRD diffractogram for MCM-48 indicates following signals: 211, 220, 400, 420, 332 and 422 corresponding to a three-dimensional (3D) structure exhibiting *la3d* symmetry. The PXRD for MCM-48/HA contain characteristic signals for MCM-48 *Ia3d* symmetry. Additionally major signals 002, 211, 130, 222 and 213 corresponding to a hexagonal crystal structure of HA exhibiting $P6_3/m$ symmetry have been recorded. FT-IR spectra obtained with MCM-48 and MCM-48/HA contain signal for molecular sieve and HA (MCM-48/HA only). Presented TEM image (Fig. 1) shows MCM-48 and MCM-4/HA composite. Structure of MCM-48 and plate like crystals of HA are shown in the picture.

DISCUSSION & CONCLUSIONS: In this study we have obtained a composite MCM-48/HA by method not previously used. The composite of MCM-48 retains the crystallinity and the resulting HA crystals are very small. As a next step we will load the cytostatic drugs into the obtained composite MCM-48/HA.

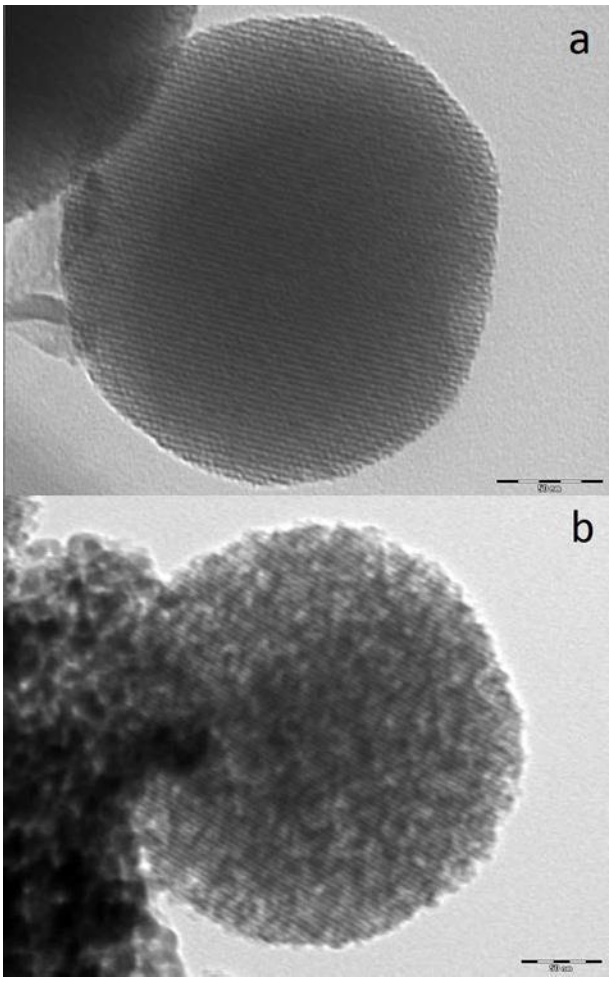

Fig. 1: TEM image of a) MCM-48, b) MCM-48/Ha nanocomposite.

ACKNOWLEDGEMENTS: *This work was financially supported by the National Science Centre (Poland), decision No. DEC-2013/11/N/ST5/02751).*

"Production and mechanical properties of nano-hydroxyapatite / polymer composites for bone defects".

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INTRODUCTION: For several years, research has been conducted in the field of regenerative medicine aimed at osteoinductive materials that are supposed to integrate with the organism or undergo resorption and be replaced by new tissue. The aim of the presented research is to produce an osteoinductive composite of nano-hydroxyapatite and biodegradable polymer. This material is similar to natural bone as regards chemical, physical and mechanical properties.

METHODS: The scope of research covers production of composite, its moulding and characterisation: microstructure, mechanical properties.

The hydroxyapatite nanopowder (nHAP) used is produced in our laboratory in the microwave solvothermal synthesis process [1] permitting modification of its nanostructure to make it fully resorbable. The other component used for producing the composite is polylactide (PLA), i.e. a biodegradable polymer commonly applied in medicine.

The composite was produced by the mechanical milling method at temp. of -190°, i.e. with increased PLA brittleness. A 6775 SamplePrep Freezer/Mill by SPEX was used for this purpose. Such a device enables mechanical shredding of substances through impacts by a ferromagnetic spindle at a temperature of -190°C. The spindle is moved inside the chamber by means of a variable magnetic field. The composite produced is milled at the frequency of 12 impacts/s, with milling time between 10 and 20 minutes.

The composite obtained is composed of heterogeneous granules containing ca. 50% of PLA and 50% of nHAP (by weight).

The composite produced was moulded by the isostatic moulding method at the pressure of 50MPa and temperature up to 165°C.

Homogeneous solid samples were obtained and subsequently tested for mechanical properties and microstructure.

RESULTS: The obtained composite - nano-hydroxyapatite with biodegradable polymer - is characterised by mechanical properties similar to natural bone, with compressive strength of 110MPa.

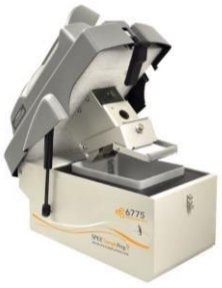

Fig. 1: Cryogenic mill

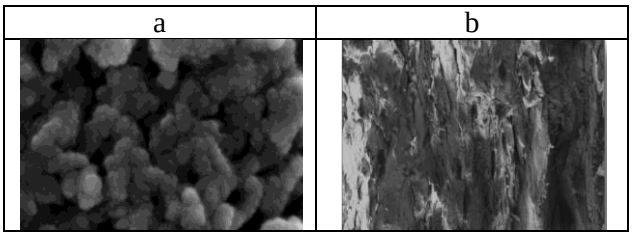

Fig. 2: Microstructure of (a)nano-hydroxyapatite, magnification x500 000 (b) composite 50% of PLA and 50% of HAP, magnification x200 .

DISCUSSION & CONCLUSIONS: The produced nano-hydroxyapatite / polymer composite is a promising biomaterial for application in orthopaedics in bioresorbable arthroscopic screws and wedges. The applied cryogenic milling method enabled obtaining a material containing 50% of PLA and 50% of nHAP.

ACKNOWLEDGEMENTS: This work was part of the project GoIMPLANT and was financed by M-era.NET.

Proteomic identification of predictive markers related with biocompatibility problems

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INTRODUCTION: The development of new materials in dental implantology is based on achieving a good implant osseointegration[1]. For that it is essential to design biocompatible materials that do not negatively interfere in the healing process[2]. After biomaterial implantation, a variety of blood proteins are adhered onto its surface. These proteins may determine the *in vivo* response to the implant by regulating, healing process, blood coagulation, inflammation and immune response[3]. Following this premise, we hypothesized that it can be possible to predict biocompatibility identifying the proteins adhered to the surface. To that end we used proteomics to analyse first layer adhered proteins and correlate the results with the biomaterial *in vivo* response. Results are discussed.

METHODS: Four distinct hybrid sol-gel materials were synthesized employing as precursors methyltrimethoxysilane (M), 3-glycidoxypropyl-trimethoxysilane (G), tetraethyl orthosilicate (T) and triethoxyvinylsilane (V) in molar ratios of 70M:30T, 35M:35G:30T, 50M:50G and 50V:50G. SLA-Ti discs were coated with prepared materials by dip-coating. *In vitro* biomaterial evaluation was assessed with MC3T3-E1. *In vivo* experimentation was carried out implanting sol-gel coated Ti dental screws in rabbit proximal tibia. Proteomic assay was conducted by incubating the discs with human blood serum for 3 h. Non-adhered proteins were removed through washes and proteins attached to the surfaces were eluted with SDS/DTT solution. Finally, eluted proteins were evaluated using mass spectrometry (LC/MS/MS).

RESULTS: Despite the composition variances, no *in vitro* differences were detected between materials. All of them showed as good behavior as SLA-Ti. *In vivo* assay showed that 70M:30T and 35M:35G:30T achieved a good osseointegration. However, 50M:50G and 50V:50G resulted in fibrous capsule formation. Proteomics were able to identify 171 distinct proteins adhered to the coatings. The comparison between coatings with different *in vivo* outcomes resulted in the identification of 9 proteins differentially more adhered to biomaterials with deficient biocompatibility. Including proteins like CRP, SAMP, C1QC or CO7. This group of proteins are related to immune complement system (Fig. 1). In fact, much of them belong to a proteomic cluster related to an acute inflammatory response.

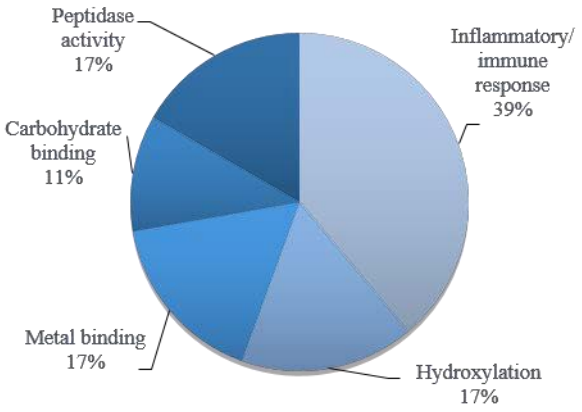

Fig. 1: Function properties of proteins adhered differentially on materials with bad biocompatibility (Anova p-value < 0.05, n=4)

DISCUSSION & CONCLUSIONS: The results suggest that the different proteomic layer composition of biomaterials with bad *in vivo* outcome could explain the fibrous capsule formation. Therefore, proteomic assays could be an important tool for biocompatibility problems prediction. In addition, a group of possible biomarkers have been identified.

ACKNOWLEDGEMENTS: This work was supported by the MAT 2014-51918-C2-2-R (MINECO), P11B2014-19, Plan de Promoción de la Investigación de la Universidad Jaume I (Predoc/2014/25), Generalitat Valenciana (Grisolia/2014/016) and Ilerimplant S.L.

Chitosan incorporated in denture base resin reduce biofilm formation of *Streptococcus mutans* and *Candida albicans*

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INTRODUCTION: Microbial colonization and biofilm formation on polymethylmethacrylate (PMMA)-based dentures may lead to denture stomatitis, which is prevalent among denture wearers[1]. Denture stomatitis is associated with multispecies biofilm formation on the denture surface[1]. There is therefore a need for denture base materials with anti-biofilm properties. Chitosan is a carbohydrate polymer derived from chitin with a reported antimicrobial effect against bacteria and fungi [2]. The aim of this study was to investigate the biofilm inhibitory effect of methacrylated chitosan covalently incorporated into the PMMA network of denture base resin against *Streptococcus mutans* and *Candida albicans.*

METHODS: Methacrylated chitosan was synthesized by reacting low molecular weight (LMW) chitosan with methacryloyl chloride under acidic conditions and characterised by proton nuclear magnetic resonance spectroscopy. The modified chitosan powder (20 wt%) was mixed with PMMA (Major) prior to addition of methyl methacrylate (Major) and homogenous sample disks were prepared. The biofilm inhibitory effect was investigated using *Streptococcus mutans UA159* and *Candida albicans* ATCC 10231. *S. mutans* was grown in BHI for 24 hours before quantification of the biofilm was performed using resazurin. *Candida albicans* was grown for 48 hours in yeast nitrogen base supplemented with 50 mM dextrose before biofilm quantification was performed using XTT. The effect of the modified material was compared to that of natural LMW chitosan. Ninhydrin was used to investigate the presence of amine groups on the surface of the material.

RESULTS: The degree of methacrylation of chitosan was 16% as confirmed by ^{1}H NMR spectroscopy. Biofilm studies showed that denture base material containing methacrylated chitosan (20 wt%) reduced both biofilm formation of *S. mutans* and *Candida albicans* compared to LMW chitosan and control. Ninhydrin detected the presence of amine groups on the surface the material.

DISCUSSION & CONCLUSIONS: Methacrylated chitosan incorporated in a PMMA-based denture resin reduced biofilm formation of *S. mutans* and *Candida albicans*. These findings show that methacrylated chitosan may be a promising candidate to be incorporated into denture base resin to reduce biofilm formation on the denture surface, which may reduce the frequency of denture-biofilm associated stomatitis.

Design of Experiments (DOE) for the development of antimicrobial chitosan conjugates

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INTRODUCTION: Design of Experiments (DOE) is a statistical method for planning and analyzing the outcome of a restricted number of key experiments that are designed to be maximally informative. DOE is used in material science and has been applied, for example, to the optimization of drug release from silicone matrix [1]. In previous studies, DOE has mainly been used to guide the composition of materials but in the current study we have, for the first time, used DOE to guide the synthesis of biopolymer conjugates and to optimize the degree of substitution for cationic, neutral and lipophilic substituents to give optimal antibacterial activity and solubility as well as minimal toxicity.

METHODS: Specialized software was used for planning and analysing the results of a DOE study (MODDE from MKS Data Analytics Solution, Umea Sweden). The experimental design was based on three variables that were the degree of substitution (DS) for three functional groups. These were the cationic *N*,*N*,*N*-trimethyl group (TRI), neutral *N*-acetyl group (ACE) and lipophilic *N*-stearoyl group (STE). These groups were introduced onto the polymer at specified DS by reaction with the corresponding acyl halide and alkyl halide in 'one-pot synthesis' utilizing the TBDMS protected chitosan as the starting material to prevent modification at the OH group (Figure 1A.). The D-optimal design matrix consisted of 14 materials with DS of 0.05–0.95 for TRI, 0.05–0.95 for ACE and 0–0.3 for STE. The effect of these parameters on the responses: log 1/MIC for *S. aureus* (y1) and *E. coli* (y2) hemolytic activity (y3) and solubility (y4) was studied and an overall SAR for the biopolymer conjugate was developed considering linear, quadratic and interaction terms.

RESULTS: The model was fitted with partial least squares optimization (PLS) .Two of the quadratic terms and all the interaction terms were found to be insignificant. Hence, a refined model was created where all the interaction terms were excluded and only the significant quadratic terms were kept in two cases. The G-efficiency and the R2 and Q2 values (y_1 = 0.76, y_2 = 0.68, y_3 = 0.77 and y_4 = 0.95) confirmed the model validity and its prediction power. The SAR could be described with four response contour plots (Figure 1B. shows two of these). An optimization showed parameters for an optimal conjugate with maximal antibacterial activity, minimal toxicity and good solubility as TRI = 0.63, ACE = 0.08 and STE = 0.29. This result was validated experimentally.

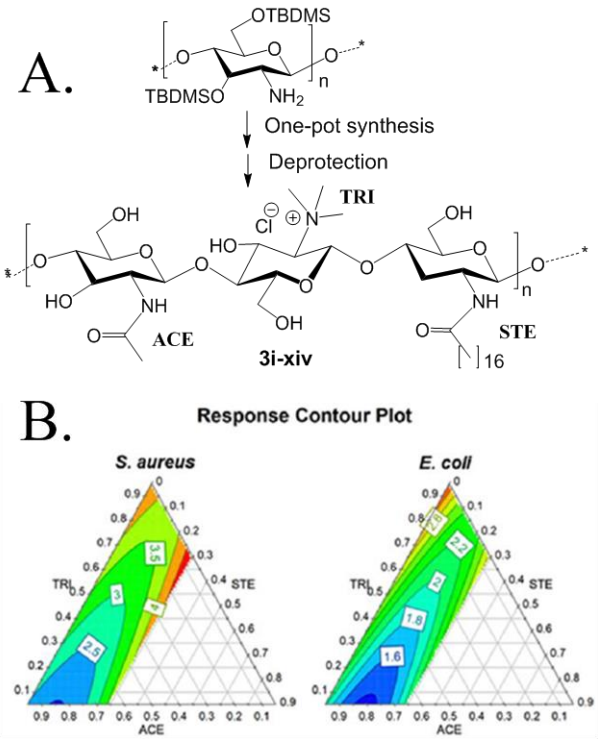

Fig. 1: (A.) The reaction scheme for the synthesis and (B.) contour plot for antibacterial activity.

DISCUSSION & CONCLUSIONS: This proof-of-concept study [2] showed that DOE is a promising approach for the optimization of complex biopolymer conjugates and SAR study of such compounds in biomaterial research.

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Acidic hydrolysis of chitosan and a derivative and the effect on antibacterial properties

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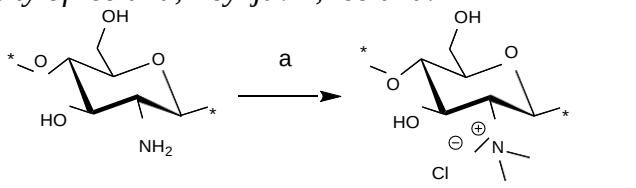
INTRODUCTION: The antimicrobial properties of the biopolymer, chitosan are known to be influenced by several factors, one of them being the chain length or the average molecular weight (Mw) of the polymer. Several studies have been performed for limited Mw range and reported different chain lengths for optimal antimicrobial activity [1, 2]. Hence, there is a need for a more systematic study over a wider Mw range to investigate the effect on antimicrobial properties.

METHODS: Chitosan polymer (TM 4138) with degree of deacetylation (DA) 21% and average molecular weight (Mw) 294 kDa was obtained from Primex ehf (Reykjavik, Iceland). Chitosan and *N,N,N*-trimethyl chitosan (TMC) was degraded by treatment with concentrated or 1M HCl at 30°C or 60°C and samples were collected at different time intervals. Degradation of the polymers was determined by measuring the Mw using size-exclusion chromatography (GPC). Characterization of the compounds was performed using ^{1}H NMR and IR spectroscopy. Antibacterial tests were performed against *Staphylococcus aureus* (*S. aureus,* ATCC 29213) using Broth Microdilution method.

RESULTS: TMC was synthesized by stirring chitosan in DMF–H_2O followed by addition of MeI and NaOH at room temperature for 48 h (Fig.1). The obtained degree of trimethylation in the polymer was calculated to be 60–70%. Acidic hydrolysis of chitosan (294 kDa) and TMC (186 kDa) was performed both under mild (1M HCl) and strong (12 M HCl) acidic conditions. The degradation resulted in chitosan with Mw varying from 294–2 kDa, and TMC with Mw varying from 186–2.5 kDa. The kinetics of degradation for chitosan showed that maximum degradation (25% in 1M HCl and 57% in 12M HCl) were obtained within 0-15 mins of treatment, and this increased upto 67% and 85% respectively at the end of 24 h. After 24 h, the rate of degradation declined significantly and prolonged hours were required to obtain the low Mw samples. For TMC, a similar



Chitosan *N,N,N*-trimethylchitosan

Fig.1: Synthetic scheme for TMC; a = MeI, NaOH, DMF-H_2O, room temperature, 48h.

rate of degradation was observed. Furthermore, the degradation with 12 M HCl was found to have 1.7–3-fold increased rate compared to 1 M HCl. The effect of temperature on the hydrolysis of chitosan using 0.1M, 1M and 12M HCl was also studied and results showed that the increase in degradation rate was 2-7-fold when the temperature was increased from 30°C to 60°C. The antibacterial activity of chitosan against *S. aureus* showed no significant variation (MIC = 4,096– 8,192 µg/mL) in the Mw range 294–10 kDa. However, for Mw <10 and glucosamine, no activity was observed with MIC values ≥ 32,768. In the case of TMC, the activity remained constant from Mw 186–20 kDa (MIC = 32-64 µg/mL), but from 10 kDa and below the activity declined and reached a minimum value at 3 kDa (MIC = 32,768 µg/mL).

DISCUSSION & CONCLUSIONS: The results showed that acidic hydrolysis of chitosan and TMC was dependent on the strength of the acid used and also on the temperature of degradation. In this study, the optimal range for chitosan and TMC for displaying antimicrobial activity against S. aureus was found to be 294–10 kDa or 186–10 kDa respectively. Below 10 kDa, the activity either declined or disappeared completely.

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Mineralised injectable hydrogels for bone regeneration

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INTRODUCTION: Hybrid, proteolytically cleavable PEG-peptide hydrogels represent a promising platform for *in situ* bone tissue regeneration[1]. For an injectable application, gelation has to occur within a critical time window, preventing the gel from being flushed out, yet allowing sufficient time for mixing of the components. Elasticity of the formed hydrogel scaffold is an important factor directing cell fate[2]. Mechanical properties were therefore assessed both on bulk- and on cellular-scale. In a next step, calcium phosphate minerals were precipitated within the gels in a one step technique to mimic a mineralised tissue environment, provide cellular attachment sites and depots for calcium and phosphate ions.

METHODS: Slate hydrogels were formed by mixing 4–armed maleimide functionalised PEG macromeres with a synthetic peptide at controlled pH. Gelation kinetics were followed by rheometry. The stiffness of fully cured, swollen gels was measured by rheometry and AFM nanoindentation. Gel architecture was assessed via mass swelling ratio and small angle X-ray scattering.

Mineralised hydrogels were produced in a similar manner. Phosphate and calcium ions were added to polymer and cross-linker phase, respectively. Mineralisation experiments were performed in different conditions, buffered (200 mM acetate buffer pH 5) or non-buffered as well as in the presence of calcite (CAL) or hydroxyapatite (HA) seeds or without seed crystals. After mixing, the mineralising gels were allowed to cure for 5 min in a flow cell before being flushed with excess Tris buffer pH 7.4. The mineralisation process was followed by phase contrast light microscopy. Phase, morphology and distribution were further examined via Raman microspectroscopy and SEM as described previously[3].

RESULTS:

Rheometry shows that gelation kinetics are controlled by the pH. Gelation takes about 3 minutes at pH 3 and 1 min at pH 4. However, network architecture and mechanical properties are not affected. All slate gels exhibit E-moduli of approximately 7.5 and 10.5 kPa on µ- and bulk-scale, respectively.

The pH drops significantly during mineralisation. However, this effect is reduced when buffer is added. Qualitatively different minerals precipitate for buffered and non-buffered systems (see Fig. 1). Also, more and smaller minerals are formed in the presence of seeds. This is especially pronounced in the case of CAL seeds.

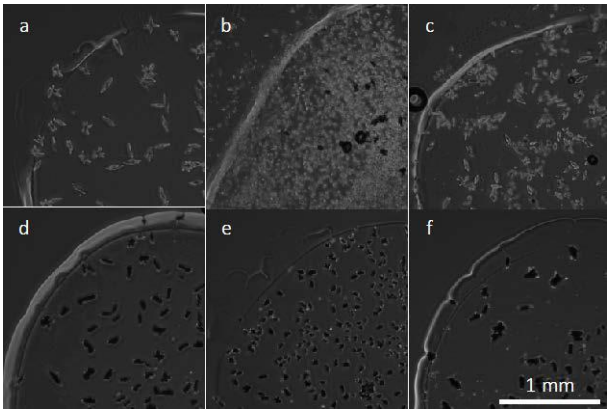

Fig. 1: Phase contrast images of mineralised gels after 20 min. Non-buffered systems without (a), with 0.1 wt.% CAL (b) and with 0.1 wt.% HA seeds. Buffered systems without (d), with 0.1 wt.% CAL (b) and with 0.1 wt.% HA seeds.

DISCUSSION & CONCLUSIONS: The performed experiments show that the gelation kinetics can effectively be controlled by altering the pH. The mineralisation process of calcium phosphate minerals releases hydrogen ions and thus causes a pH drop. Though, when increasing or buffering the initial pH, gelation still takes place in a fast and controlled manner. Homogenous distributions of mineral were obtained for all tested conditions. Phase, size and morphology of the minerals can effectively be controlled by pH and seed crystals.

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Phosphorylation modulate AMELOBLASTIN self-assembly and Ca^{2+} binding

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INTRODUCTION: During tooth development ameloblastin (AMBN) is secreted into the mineralising matrix in a complex interplay between self-assembled proteins and ions that is not well understood. Regions encoded by the exon-5, are required for self-assembly of AMBN into fibrils (1). Phosphorylation of secreted enamel proteins, like AMBN, may contribute by positioning of calcium (Ca^{2+}) and phosphate during amelogenesis. Whether phosphorylation modulates self-assembly of AMBN is however not known. AMBN carboxyl terminal (C-term) contains intrinsically disordered protein regions (IDPR), predicted to bind Ca^{2+} (2) that may also be phosphorylated even when AMBN is in its fibril state.

METHODS: Recombinant full-length AMBN (AMBN-WT) and its constituent peptides (N-term, C-term, an exon-5 deletion variant (DelEx5), and various N-term peptides) were phosphorylated by recombinant casein kinase 2 (CK2) and protein kinase A (PKA), separated and analysed with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The disorder of the AMBN protein/peptides was analysed with Small Angle X-ray Scattering (SAXS).

RESULTS: Self-assembled, fibril AMBN-WT showed a high degree of order and did bind Ca^{2+}. Self-assembly reduced the phosphorylation by PKA compared to peptides translated from exon 2-4 and exon-5. The disordered C-term remained monomeric, and failed to bind Ca^{2+} at higher concentrations. The C-term was phosphorylated both the monomeric and as part of AMBN-WT by CK2 and PKA. In contrast DelEx5 was disordered, formed dimers, failed to bind Ca^{2+}, and was hardly phosphorylated in the C-term region.

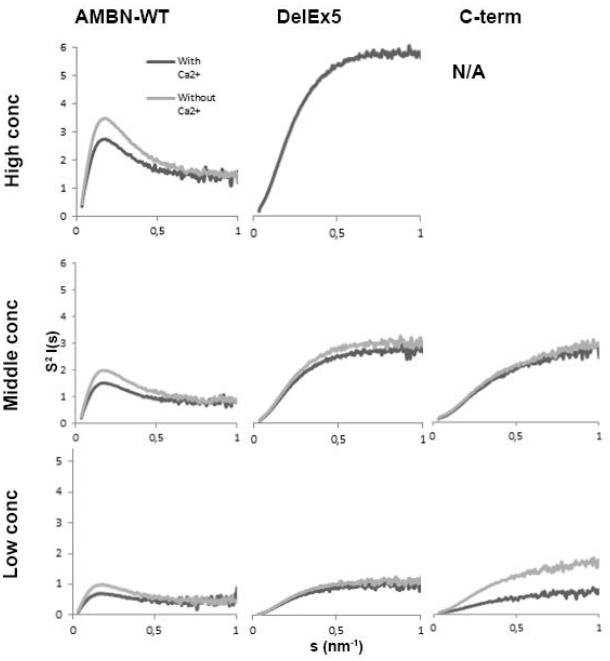

Fig.1 Small angle X-ray scattering show influence of $CaCl_2$ on structure

DISCUSSION & CONCLUSIONS: The intrinsic disordered regions within AMBN-WT C-term seem to allow phosphorylation by CK2 while self-assembly seem to occupy phosphor acceptor sites in the more structured N-terminus part of the molecule. Furthermore, Ca^{2+} binding has preference for the fibril form of AMBN. Additionally, phosphorylation of exon-5 by PKA, hinder self-assembly and modulate Ca^{2+} binding. In conclusion, the results suggest that phosphorylation of AMBN could be an important regulatory mechanism in enamel matrix assembly and mineralisation.

1.

Bioinspired polyphenol nanocoatings for bioactive bone-anchored implants

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INTRODUCTION: Polyphenol coatings have recently attracted increasing interest as surface functionalisation strategies for medical implants due to their diverse functional properties: these coatings have been shown to possess antioxidant, anti-inflammatory, antibacterial, and antifibrotic properties [1-4]. The objective of this study was to assess the coating deposition of two different phenolic compounds, tannic acid (TA) and pyrogallol (PG) on titanium surfaces, and the effect of these coatings on biofilm formation and osteoblast adhesion and differentiation.

METHODS: Human primary osteoblasts were cultured on mirror-polished titanium samples with polyphenol coatings deposited for 2 h (TA2, PG2) and 24 h (TA24, PG24), while uncoated samples served as control. Cytotoxicity and metabolic activity were evaluated after 48 h, whereas alkaline phosphatase (ALP) activity and calcium content were measured after 21 d. Gene expression of osteocalcin (OC) and ALP was assessed with qPCR. *S. epidermidis* was used to assess bacterial adhesion and biofilm formation of the deposited polyphenol coatings. Growth and viability of bacteria seeded on the samples were assessed by quantifying both planktonic and adherent bacteria (OD and CFU). In addition, the formed biofilm was imaged using SEM and confocal microscopy (live/dead staining). Stability of the formed polyphenolic coatings was assessed with QCM-D.

RESULTS: Regardless of their thickness, both tannic acid and pyrogallol coatings were found to be non-toxic to human primary osteoblasts and allow cell adhesion to the coated implant surface. Thinner coatings deposited for 2 h (TA2 and PG2) were shown to be more osteogenic with higher calcium content and ALP activity at 21 d in comparison to both the uncoated Ti surface and coatings deposited for 24 h (TA24 and PG24). Gene expression of OC and ALP showed similar results with TA2 and PG2 showing increased mRNA expression in comparison to the corresponding 24 h polyphenol coating.

While biofilm formation was not affected by the polyphenol coatings, both the OD and CFUs of planktonic bacteria cultured in the presence of samples with coatings deposited for 24 h (TA24 and PG24) were significantly reduced in comparison to both 2 h coatings and uncoated Ti.

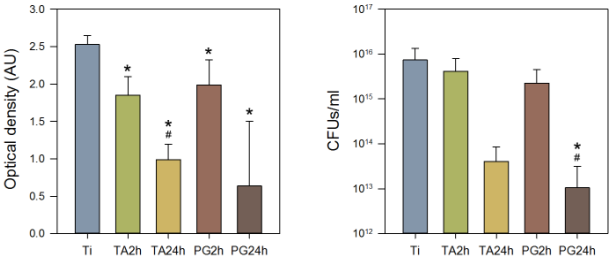

Fig. 1: Optical density and colony-forming units for planktonic bacteria after 12 h exposure to polyphenolic compounds released from the coatings

Apart from PG2, a substantial frequency increase was observed with QCM-D when coated surfaces were rinsed with PBS for 12 h, indicating release of phenolic compounds from the surface.

DISCUSSION & CONCLUSIONS: Tannic acid and pyrogallol formed homogeneous coatings on titanium surface by auto-oxidation mediated polymerisation. Both TA and PG coatings on Ti promoted osteogenic differentiation of osteoblasts adhered onto the samples surface, particularly on coatings formed at short deposition times resulting in thinner and more rigid phenolic coatings. The reduction in osteogenic differentiation on coatings deposited for 24 h may result from their higher hydration ratio and altered mechanical properties. Furthermore, release of the phenolic molecules from the coated surface may influence cell behaviour with higher concentrations resulting in delayed growth and differentiation of the cultured cells. Released phenolic compounds were also found to reduce the planktonic growth of *S. epidermidis*. Unlike previously reported [2], the formed polyphenol coatings did not result in contact killing of *S. epidermidis* adhering to tannic acid coated surfaces.

ACKNOWLEDGEMENTS: This study was supported by the Norwegian Research Council (Grant 230258).

Investigations on the tailorability of hard tissue implant surfaces by printing

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INTRODUCTION: The demand for improved implant performance is placing manufacturers in a position to reassess their processing capabilities and use advanced characterization techniques to explore further characteristics and properties. Thermal spraying is paving the way to a potentially new process for tailored surfaces. Printing through a thermal source offers unique characteristics that cannot be provided by other processes, especially if particles are molten and then resolidified; crystallinity directly impacts the bioactivity and the less commonly measured surface potential can influence cell proliferation. Following the theme of the Scandinavian Society for Biomaterials conference on "Underlying challenges in Biomaterials" this work will investigate the change in printed droplet characteristics and see how the geometry, topography, crystallinity and surface potential change with spray distance.

METHODS: A narrow hydroxyapatite particle size powder (25-44 μm) was sourced from CAM Bioceramics and flame sprayed onto 100 °C preheated polished titanium discs at spray distances of 4, 8, 12, 16 and 20 cm.

Characterization tools were chosen to assess the topography, phase and electrical potential. The flattened droplet size was measured by optical microscopy and a map of droplets produced to assist the identification of separate splats. The shape was further investigated with atomic force microscopy. Phase was investigated by micro X-ray diffraction and supplementary information obtained by Fourier transform infra-red spectroscopy and microRaman spectroscopy. Electrical potential across each droplet surface was evaluated with Kelvin probe AFM.

RESULTS:

X-ray diffraction of the prints made at a spray distance of 4 cm displayed diffraction peaks suggesting a crystalline structure, however, the lack of diffraction peaks for samples made at larger spray distances suggested an amorphous phase. Raman spectroscopy did not show any signal indicating a lower crystallinity material, but FTIR-DRIFT showed absorption indicative of low crystallinity.

The shape of the droplets at shorter spray distances represent a "doughnut-like" shape, but longer spray distances showed a transition to a hemispherical shape, Fig 1.

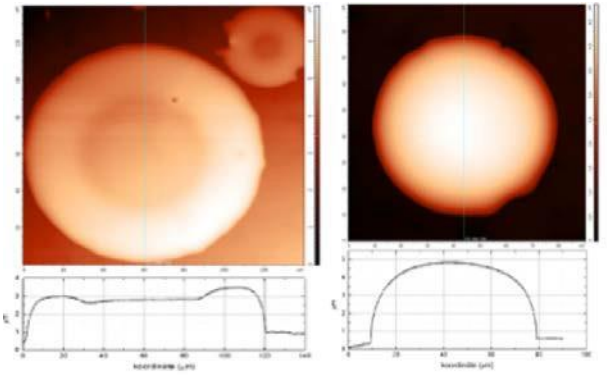

Fig. 1: Droplet topography measured by AFM for a splat produced at 8 cm and 16 cm.

The electrical potential displayed variation over the range of spray distances. At a spray distance of 4cm, the electrical potential was the highest with an average of 1mV. At longer distances, the potential was lower, but after a sudden decrease to 0.1 mV then slowly increased to 0.5mV.

DISCUSSION & CONCLUSIONS: Splats displayed a change in shape and electrical potential with spray distance. Both of these aspects have not previously been reported. At a short spray distance, a rim formed from recoil of the spread droplet [1], but at longer spray distances a hemispherical droplet resulted. The electrical potential was largest for the crystalline phase. For the amorphous phase, the electrical potential was lower when the splat was formed at a lower spray distance. This could have arisen from the change in hydroxyl ion content in the splats. This work provides new insight on the shape and electrical potential of splats.

ACKNOWLEDGEMENTS: Projects "Refined Step" and "Signaling Implant" have contributed to the outcome reported here.

Following the mineralization of hydrogels

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[2]*Department of Biomaterials, University of Oslo, Norway.*

INTRODUCTION:
Natural bone tissue is a highly complex structural composite in which the main load bearing functions are performed by a highly mineralized collagen-based material. Bone is produced in a process with a high degree of biological control, in various stages of development, as well as in response to injury or changing mechanical requirements. Bone is a dynamic material, constantly repaired and remodeled by a range of bone-resident cells. In the context of tissue engineering (TE), attempts are being made to make artificial composite materials which resemble bone and which, in combination with biological factors, could enhance bone tissue regeneration or replace bone fragments. Various approaches are researched, including organic inorganic composites, ceramics, biodegradable polymer or metals. Hydrogel-based composites offer several advantages, as they can be made injectable, are biocompatible and can easily be combined with cells and/or mineral precursors. Mineralization of hydrogels can provide a useful method to make materials which are cell compatible, multicomponent, and provide simple means of cell and mineral precursor transport and therefore enhance regeneration. We have described a system in which hydrogel mineralization processes can be studied in a correlative manner with a range of experimental methods. In particular we have focused on microscopy and spectroscopy and investigated mineralization and mineral phase transformation of alginate/CaP composites.[1] Here we show how the same approach can be used to study and optimize mineralization of collagen gels and synthetic PEG-peptide hydrogel systems.[2]

METHODS: All hydrogel mineralization experiments were conducted in a pseudo-2D geometry, where a thin slab of hydrogel was created between two glass slides. For collagen gels, mineralization was done by diffusion of $CaCl_2$ solution into a preformed collagen gel disk containing phosphate ions. For two component peptide cross-linked PEG gel, mineral was formed during preparation of the gel, and the composite was moulded into a disk shape before the gelling was completed.

RESULTS: The experimental design allows us to follow mineral formation in the hydrogel and to optimize conditions to achieve desired mineralization. For collagen gels, we can investigate how a growing mineral phase interacts with the gel network (Fig 1) and investigate the presence of metastable CaP-phases during the mineralization process. Octacalcium phosphate was detected in early timepoints which later transformed into the more stable hydroxyapatite. For PEG gels, we study how mineralization conditions influence the mineral precipitation and transformation and optimize conditions for making hydrogels with well dispersed, nanoscale phase of CaP.

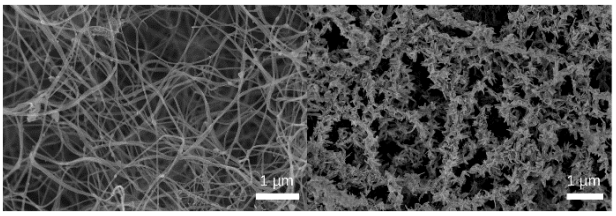

Fig. 1: SEM micrographs showing unmineralized (left) and mineralized (right) collagen gels.

DISCUSSION & CONCLUSIONS:

We show that by combining a simple, but powerful experimental setup with several correlative advanced characterization techniques, new insight into the mineralization processes within hydrogels can be obtained. This insight can be applied to optimize fabrication strategies for injectable hydrogel systems for bone TE. Preliminary experiments with collagen and PEG gels show that this approach could be used to study the role of additives as well as mineralization strategy to make hydrogel/CaP composites with desired characteristics.

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Calcium phosphates with high specific surface area towards improved cell response *in vitro*

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INTRODUCTION: Specific surface area (SSA) is an important property of any material, including biomaterials. It is a well known that higher SSA ensures better solubility and reactivity compared to the same material with lower SSA.

Calcium phosphates (CaPs) are widely studied for use in bone replacement, repair and regeneration as they resemble the mineral part of the bone tissue.

CaP scaffolds at the market have relatively low SSA (0.5-2 m^2/g) [1-3] while bone mineral has SSA of 87-100 m^2/g [4]. Therefore, in many cases autologous bone grafts are still preferred over synthetic grafts, regardless of disadvantages of former ones. Higher SSA of synthetic scaffolds would approach set of properties of bone mineral.

Current study focuses on cell response *in vitro* of calcium phosphate (CaP) scaffolds with different SSA (high and low).

METHODS: *In vitro* cell behaviour was tested on two types of CaP scaffolds: ones with high SSA of 78±5 m^2/g (designated as A), others with lower SSA of 28±2 m^2/g (designated as B). Differences in SSA of CaP scaffolds were obtained by different heat treatment (HT) regimes – lower HT temperature for higher SSA and higher HT temperature for lower SSA. Characterization of scaffolds was done with XRD, FT-IR, SEM and BET N_2 adsorption methods. For *in vitro* tests MG63-GFP cells were used. Number of cells was determined by use of fluorescent microscopy. Attachment of fixed cells was observed with SEM as well. Each series had 6 parallel samples.

RESULTS: Main characteristics of tested scaffolds are shown on Table 1.

XRD analysis revealed that A scaffold was composed of amorphous calcium phosphate (ACP); B scaffold was crystalline and composed of beta tricalcium phosphate (β-TCP) with admixture of hydroxyapatite (HAp).

FT-IR spectra confirmed presence of chemical groups characteristic to phases identified with XRD. Additionally, carbonate groups (CO_3^{2-}) were detected for both scaffolds. Higher HT temperature decreased amount of CO_3^{2-} groups.

In vitro cytotoxicity results showed that better cell viability (77%) was for A scaffolds with higher SSA comparing to positive control. B scaffolds gave lower cell viability (29%).

Table 1. Characteristics of studied samples, where CV – cell viability.

	SSA, m^2/g	Phase	CV, %
A	78±5	ACP	77
B	28±2	β-TCP, HAp	29

DISCUSSION & CONCLUSIONS: CaP scaffolds with higher specific surface area demonstrated 2.7 times better cell viability *in vitro* than ones with lower specific surface area. However, observed differences might be related to differences in phase composition and amount of carbonates as well.

Further studies should be devoted to separate possibly overlapping effects of specific surface area and differences in phase compositions.

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Human adipose stem cell osteogenic differentiation in 3D hydrogels

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INTRODUCTION: There is a pressing demand for engineered bone grafts due to lack of suitable donors and in relation to traumas and increasing ageing population. Human adipose stem cells (hASCs) are an abundant and accessible source of adult stem cells and suitable for the development of bone constructs [1]. 3D hydrogel culture simulates better cellular microenvironment compared to traditional biomaterials. This study proposes an *in vitro* model for osteogenic differentiation of hASCs in 3D hydrogels.

METHODS: The gellan gum (GG) and collagen type I (COL1) (rat tail) hydrogels were tested for their ability to support hASC viability and ability to spread in 3D hydrogel culture. Cell proliferation based on total DNA content was quantified with CyQUANT analysis. Gene expression of osteogenic marker genes *ALP*, *OSX*, *DLX5*, and *RUNX2A*, and quantitative alkaline phosphatase activity (qALP) were analyzed to determine early osteogenic differentiation of hASCs. Late osteogenic differentiation of hASCs was analyzed based on the mineralization of the hASC ECM measured by hydroxyapatite formation and by immunofluorescence staining of bone marker protein osteocalcin. Different types of mineral residues in different hydrogels were analyzed by Raman spectroscopy. Optical projection tomography (OPT) [2] was used to image hASCs encapsulated in hydrogel.

RESULTS: The hASCs were well viable in 3D hydrogel culture. GG encapsulated hASCs had tight and round cell morphology, whereas in COL1 hydrogel the cell morphology was more elongated and spread out. Overall, the qALP results were low. COL1 hydrogel showed significantly higher osteogenic marker gene expression. All the hydrogel conditions supported the formation of mineralized hydroxyapatite residues (Fig. 1), whereas strong osteocalcin staining was visible only in COL1 hydrogel.

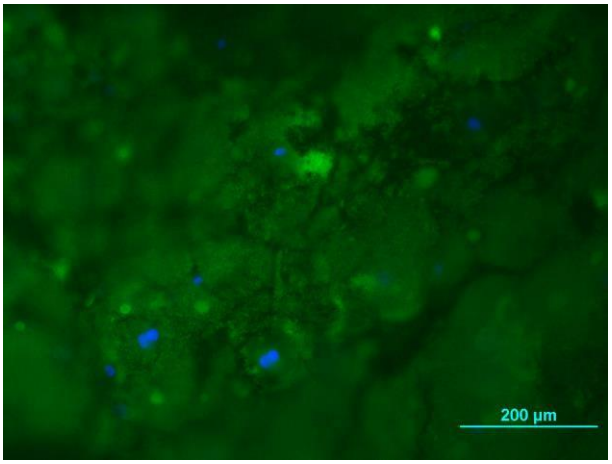

Fig. 1: Mineralization. Hydroxyapatite (green) and DAPI (cell nuclei; blue) staining of hASCs in GG hydrogel at 21 days. Imaged with epifluorescence microscope. Scale bar 200 µm.

DISCUSSION & CONCLUSIONS: These preliminary results demonstrated the potential of the 3D hydrogel culture methods to induce differentiation of the hASCs towards bone-like cells and to develop an *in vitro* model for applications of engineered bone constructs.

ACKNOWLEDGEMENTS: The authors wish to thank for technical support laboratory technicians Anna-Maija Honkala, Miia Juntunen, MSc, and Sari Kalliokoski. This study was financially supported by the Finnish Funding Agency for Technology and Innovation (TEKES) and the Human Spare Parts Project.

Modular 3D cell niches: Optimisation of biochemical and biophysical properties for bone marrow stromal cell adhesion and survival

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INTRODUCTION: Highly modular hydrogels are under development for analysis of how complex mechanotransductive processes impact on stem cell differentiation in three dimensions (3D). In two-dimensional (2D) models, cell morphology, extracellular matrix stiffness, degradability and adhesion ligand presentation play an important role in directing diverse cellular behaviours[1]. Cells encapsulated in 3D hydrogels show a similar response to stiffness, but relationships between cell morphology and differentiation are more complex in 3D than in 2D and remain poorly understood. In addition, achieving controlled presentation of cell adhesion ligands has proved to be challenging in 3D, whilst the stiffness of an enzymatically degradable matrix changes as cells rearrange their environment. These complex issues make it difficult to elucidate the individual and combined effects of different matrix properties.

The purpose of these modular hydrogels is to enable variation of individual properties with minimal impact on other characteristics of the niche. This should enable determination of both individual and combinatorial effects of various matrix properties by ensuring that introduced features, such as ligands, do not *replace* cross-linking peptides, but are instead branched from them. In addition, the use of two different chemistries via sequential reactions enables the pre-formation of ligand "clusters", lending greater control over spatial presentation of biomolecules in 3D than has previously been achieved.

METHODS: Custom synthesised degradable/non-degradable and/or adhesive/non-adhesive peptides (Peptide Protein Research Ltd., UK) were first deprotected using triethylamine in dimethyl-sulfoxide and then conjugated to four-arm PEG–nitrophenyl carbonate (synthesised according to ref. 2) at different proportions, via N-terminal amine groups (Lys). Disulphide bridges between peptides were cleaved using *DL*-dithiothreitol, prior to three ethanol rinse and centrifugation steps and lyophilisation. Hydrogels were then formed via addition of four-arm PEG–vinyl sulfone via the C-terminal thiol group (Cys) of the peptides in 50 mM HEPES pH 8.2, together with human bone marrow-derived stromal cells. Gels were optimised to enable efficient cell spreading and survival (assessed using live/dead assay, total DNA quantification and immunohistochemical staining of F-actin combined with confocal laser scanning microscopy) in hydrogels with varying stiffnesses (by varying PEG MW), adhesion ligand density or degradability (by varying peptide sequence).

RESULTS: This sequential approach to synthesising hydrogels via two different reactions resulted in successful formation of hydrogels with varying adhesiveness and degradability, which in turn affected cell spreading and survival. It was confirmed that cells require a certain level of adhesiveness and degradability in order to be able to spread and survive when encapsulated in 3D.

DISCUSSION & CONCLUSIONS: The unprecedented modularity afforded by this sequential synthesis enables formation of ligand clusters in 3D, the biological characterisation of individual matrix characteristics with minimal impact on other properties, or the combined analysis of multiple properties in parallel. Future work will focus on analysis of differentiation down multiple lineages, including neuro-, adipo-, myo-, chondro- and osteogenesis, via gene expression analysis and further immunohistochemical stains.

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Directed differentiation of pluripotent stem cells into insulin-producing islets in a macroporous hydrogel system: a pilot study

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INTRODUCTION: In this study, a micro-cavity gel (MCG) platform is adopted to provide a one-step encapsulation protocol in which pluripotent stem cells can proliferate and rapidly form EBs within prior to pancreatic differentiation, unlike conventional two-step procedures of firstly forming EBs and subsequently encapsulating them in a biomaterial scaffold. The MCG platform is a macroporous alginate hydrogel formed with the help of incorporated sacrificial gelatin microspheres, increasing space and diffusion throughout the macro-sized construct for efficient cell proliferation [1] followed by pancreatic differentiation.

METHODS: 10^7 cells (mESC and miPSC) were separately suspended per 1 mL of 4 ℃ 1.5% w/v alginate solution. The cell suspension was subsequently mixed with 0.3 g gelatin microsphere porogen (150 – 180μm diameter) per 1 mL of alginate solution. 80 μL of the mixture was transferred to each circular mold of 5 mm diameter. 102 mM CaCl2 was added onto the construct and replaced in 4°C for 8~10 min. Finally, constructs were transferred to 24-well agarose-coated plates containing cell culture medium and maintained at 37 °C, 5% CO2. Gelatin microspheres dissolute at 37 °C to leave behind pores of corresponding diameters within two days, forming a MCG system. Constructs were maintained in pluripotent cell culture medium for 10 days to allow proliferation and EB formation. Thereafter, cells were firstly induced towards definitive endoderm by culture in serum-free RPMI 1640 medium supplemented with 1× B27 supplement and 100 ng/mL Activin A for 5 days. Subsequently, for pancreatic commitment, medium was replaced with serum-free RPMI 1640 medium supplemented with 2 μM retinoic acid, 0.5% BSA & 1×ITS for 2 days. In the third step for endocrine commitment, medium was changed to DMEM/F12 supplemented with 10 ng/mL murine bFGF, 20 ng/mL murine EGF, 0.2% BSA & 1× ITS for 3 days. Finally, for pancreatic islet maturation, medium was changed to DMEM/F12 supplemented with 10 ng/mL murine bFGF, 10 mM nicotinamide, 0.2% BSA & 1×ITS for 5 days.

RESULTS: Pluripotency markers Oct4 and Nanog showed a general decreasing profile after differentiation was induced. Definitive endoderm marker Sox17 was highly expressed from Day 5 onwards and peaked at Day 7. As cells committed to a pancreatic lineage, its expression decreased. In the second stage of stepwise differentiation by Day 7, successful commitment to the pancreatic lineage is determined by Pdx1, Nkx2.2, Nkx6.1 and Gata4 expressions. In the third differentiation step for pancreas endocrine induction by Day 10, specific markers Ngn3, Pax4 and Pax6 were expressed, while Pdx1, Nkx2.2 and Nkx6.1 are downregulated. Gene expression profile of mESCs and miPSC samples continued to indicate differentiation and maturation towards β-cells by Day 15, with the high Pax4 expression levels and recurrence of high Pdx1, Nkx6.1 and Nkx2.2 levels. Finally, both mESC and miPSC samples highly expressed all markers at Day 15, but gene expression levels of miPSCs were visibly of a lower extent. At D15, Insulin (Ins1 and Ins2) mRNA levels were respectively 27.9±2.6 and 62.9±10.7 folds of control Day 0, while Glut2 was also significantly higher than control in mESC samples, indicating an inclination towards mature β-cell phenotype. This was further supported by the maintenance of high Pax4, Nkx6.1 and Pdx1 expression levels representative of mature β-cells. Immunohistochemistry stains supported gene expression trends, and showed that mESCs were more responsive to the stepwise differentiation protocol towards β-cell lineage than miPSCs. However, although insulin-secreting pancreatic cells were successfully obtained from mESCs via the stepwise differentiation protocol, they did not function well as glucose-responsive β-cells yet.

DISCUSSION & CONCLUSIONS: In this work, a 3D MCG system was used to encapsulate murine pluripotent stem cells and differentiated into pancreatic β-cell islets after EB formation in a 25-day process. mESCs were differentiated into insulin-producing cells as evaluated from its genetic and protein secretion patterns.

SiO_2 nanoparticle grafted surfaces as a model material for studying protein adsorption using acoustic sensing and circular dichroism

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INTRODUCTION: The surface chemistry and topography influences what happens when a foreign material enters the body and meets blood and tissues. Nanotechnology and the development of nanomaterials have resulted in the opportunity to design tailor made nanostructured implants with novel topographical and chemical surface properties. However, knowledge of how the topography at the nanoscale, e.g. curvature, affects protein adsorption and further the fate and success of tissue integration is still limited.

The aim of this study was to investigate how the size and curvature of nanofeatures affect the adsorption of blood proteins. By developing a model material, constructed using APTES-functionalization and SiO_2 nanoparticles of different sizes, a large spectrum of curvature with homologous chemistry can be systematically studied and analyzed. Protein adsorption will be studied *in situ* using the acoustic sensor technique Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D). Circular Dichroism (CD) will also be employed to study conformational changes of the protein secondary structure using a holder with stacked functionalized substrates.

METHODS: Silica substrates were functionalized with 3-aminopropyl triethoxysilane (APTES) using solution phase deposition in toluene [1] prior to deposition of SiO_2 nanoparticles. The Silica colloids were a gift from Akzo Nobel AB and received in four different types/sizes: 9 nm (Bindzil 360/30), 25 nm (Bindzil 130/40), 40 nm (Levasil 100/45) and 80 nm (levasil 50/50). Nanoparticle deposition was tuned by altering the ionic strength and pH of the nanoparticle dispersant. Finally, the substrates were sintered to remove excess APTES and amine groups and ensure a chemically homogenous surface.

RESULTS: Nanoparticle deposition using 0.1 mM PBS as dispersant is demonstrated in Fig. 1 followed by some preliminary results of albumin adsorption in Fig 2.

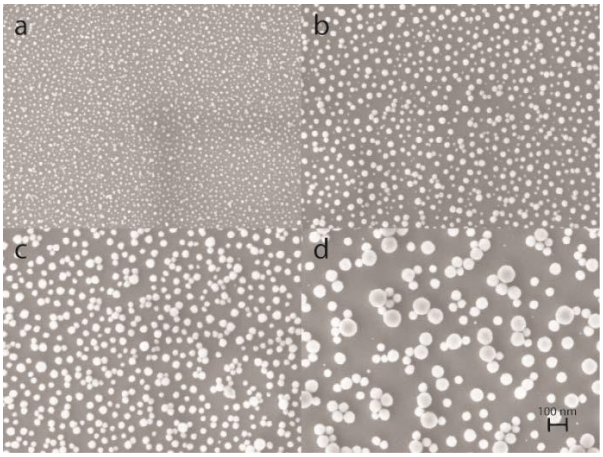

Fig. 1: Scanning electron microscopy images of SiO_2 nanoparticles deposited onto APTES functionalized silica substrates using 0.1 mM PBS as dispersant. 9 nm (a), 25 nm (b), 40 nm (c) and 80 nm (d). Scalebar: 100 nm.

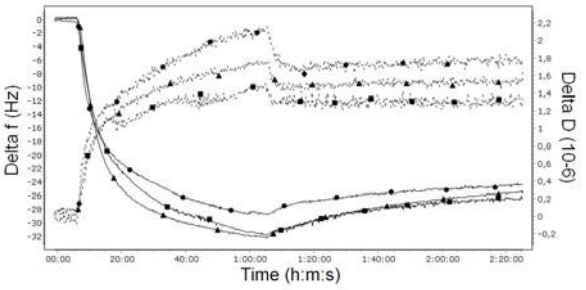

Fig. 2: Observed frequency and dissipation shifts upon 1 mg/ml albumin adsorption onto SiO_2 np grafted surfaces. 9 nm (●), 25 nm (■), 40 nm (▲).

DISCUSSION & CONCLUSIONS: Preliminary results of albumin adsorption using QCM-D indicate that adsorption is affected by the size of the nanoparticles. More results and a thorough discussion will follow later.

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Fenton-like behaviour of sol-gel coated TiO_2 scaffolds

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INTRODUCTION: It has recently been shown that TiO_2 particles can act as Fenton-like catalysts and decompose H_2O_2 in the absence of light [1]. The catalytic activity was related to the formation of superoxide radicals, which formed primarily on anatase surfaces. The aim of this study was to translate this Fenton-like behaviour to highly porous TiO_2 scaffolds used for bone repair. A sol-gel coating was developed in order to modify the crystal structure of the substrate. Further, the catalytic activity and the biological response towards modified surfaces were assessed.

METHODS: Highly porous scaffolds were fabricated using the polymer sponge method [2]. Substrates were dip coated in a titanium isopropoxide-based sol and dried at room temperature for 24h. Deposited films were calcinated at 500°C for 1h. Coatings were characterized by SEM, XRD, ellipsometry and profilometry. The catalytic activity was quantified by the degradation of methylene blue on TiO_2 scaffolds in the presence of 3% H_2O_2 and for scaffolds pre-treated with 30% H_2O_2. Further, *in vitro* studies with MC3T3 pre-osteoblasts (confocal microscopy, LDH, comet assay) and biofilm forming *S. epidermidis* (Live/dead, CFU, luminescence assay) were conducted.

RESULTS: Sol-gel dip coating of porous scaffolds resulted in a thin (41.3 ± 0.11 nm) and homogenous anatase film that did not alter the microstructure of the substrate (Fig.1).

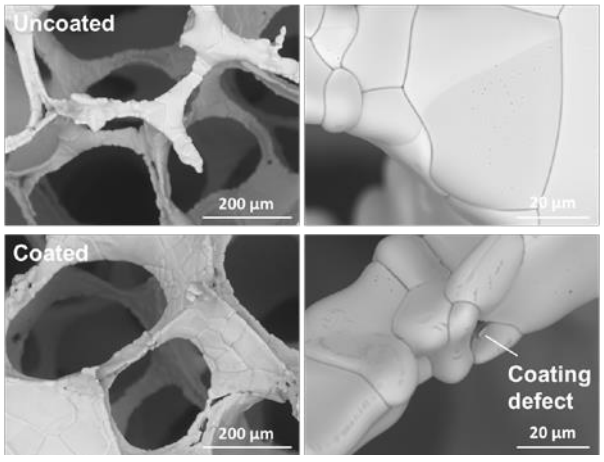

Fig. 1: SEM images of uncoated and sol-gel coated TiO_2 scaffolds.

Sol-gel coated scaffolds showed a significantly higher catalytic activity compared to uncoated scaffolds in the presence of H_2O_2 and for scaffolds pre-treated with H_2O_2 (Fig. 2). Preliminary *in vitro* results did not show any cytotoxic or genotoxic effects for pre-treated scaffolds on MC3T3 pre-osteoblasts. Both, uncoated and coated TiO_2 scaffolds, pre-treated with H_2O_2 lowered the viability of *S. epidermidis* biofilms.

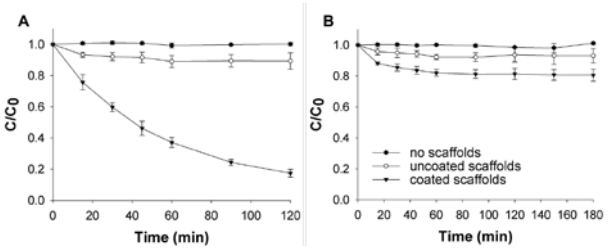

Fig. 2: Methylene blue degradation (A) at the presence of H_2O_2 and (B) for pre-treated scaffolds.

DISCUSSION & CONCLUSIONS: The newly developed sol-gel coating resulted in the desired anatase structure on TiO_2 scaffolds. The high catalytic activity of modified scaffolds at the presence of H_2O_2 is likely due to the formation of radical intermediates during the decomposition of H_2O_2 at the metal oxide surface. These radicals may also play an important role in the formation of oxidative titanium peroxo complexes formed at pre-treated surfaces [3]. Early results indicated that this surface modification was not cytotoxic or genotoxic to MC3T3 cells but may cause oxidative stress related death in *S. epidermidis* biofilms. The current findings indicate that Fenton-like chemistry is a promising anti-infective strategy for biomaterials.

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Hydrazone cross-linked hyaluronan-poly(vinyl alcohol) hydrogels for 3D neuronal applications

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INTRODUCTION: Human pluripotent stem cell (hPSC) –derived neural cell types are seen to potential source for transplantation therapies e.g. for spinal cord injury. Moreover, those cells can be used for in vitro models such as disease modelling, like Parkinson disease, and drug testing. Nevertheless, only a little is known about the ability of those cells to form 3D networks[1]. Although, the importance of *in vivo* like environment is seen widely in those applications. Thus, in this study, hyaluronan (HA)- poly (vinyl alcohol) (PVA) hydrogels are studied as 3D scaffold for hPSC-derived neuronal cells.

AIMS: Here, the aim of the study is to compare different compositions of HA-PVA hydrogels for 3D environment of hPSC-derived neuronal cells.

METHODS: Cell source of this study is both hESC and hIPSC -derived neuronal cells. Cells are maintained and cultured according to protocols published earlier [2,3].

Hydrogels were crosslinked via hydrazone crosslinking using aldehyde-modified HA component and hydrazide modified PVA component. Different concentrations of HA-PVA gels were tested.

Two different cell seeding strategies were used. First, cells were plated on top of the hydrogel and cultured for two weeks. Secondly, hydrogel components were mixed together with cell suspension in 10% sucrose solution in cell culture well. Cells were cultured as encapsulated into hydrogel for two to four weeks. After culturing period, neuronal networks were analyzed using phase contrast microscopy and immunocytochemical stainings.

RESULTS: The culturing of HPSC-derived neuronal cells was successful on top of and inside the HA-PVA hydrogels. Cells maintained the neuronality and they seem to favor softer, e.g. less concentrated hydrogels. In those hydrogels, cells formed good neural network inside the hydrogel.

DISCUSSION & CONCLUSIONS: Hydrazone cross-linked HA-PVA hydrogel offers a good solution to combine tissue mimicking natural polymer and synthetic polymer with good mechanical, thermal and chemical stability. Hydrogels tested in this study offer a stable enough (at least 4 weeks) 3D culture environment for hPSC derived neuronal cells. Thus, those gels seem to be potential 3D scaffold for hPSC-derived neuronal cells.

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Strontium and fluorine co-substituted hydroxyapatite for tooth enamel remineralization

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INTRODUCTION: Every day the hard tissue of teeth are subjected naturally occurring processes – remineralization and demineralization. The maintenance of balance between those processes is essential for health of teeth. But usually demineralization takes over remineralization due to bed habits, increased carbohydrates consumption and stressed lifestyle. Therefore the purpose of this study is to develop strontium and fluorine co-substituted calcium deficient hydroxyapatite (SrFCDHAp) nanoparticles as remineralization agent, added in dentifrices and toothpastes, to prevent loss of minerals and caries development.

METHODS: SrFCDHAp was synthesized *via* modified wet precipitation method combined with pH cycling, according to J. Rimsa *et al*. [1]. Model toothpaste was produced from 2-hydroxyethylcelluloce (HOECel), glycerol, deionized water and paste-like SrFCDHAp [2]. 6 bovine enamel fragments were used during *in vitro* enamel remineralization. Experiment lasted 7 days. To perform demineralization, the surface of specimens was subjected to treatment with 35% H_3PO_4 for 5 minutes. All samples were brushed with model toothpastes twice a day for 3 minutes. The enamel blocks were placed in the artificial saliva, between treatment episodes under dynamic condition in the incubator at 37°C. The surfaces of demineralized and remineralized samples were observed with scanning electron microscope (SEM) and elemental analysis was done with energy- dispersive X-ray analysis (EDX). The thickness of remineralized layer was evaluated with prophylometry. The results were compared to model tooth paste containing unsubstituted CDHAp.

RESULTS: SEM revealed new layer formation on the demineralized enamel surface. Fig. 1 B demonstrates the layer formed from oriented, elongated particles, while in D image the orientation of particles in the new layer was not observed. EDX analysis detected Sr, F, Ca, P and O presence in the remineralized surfaces. The measurements of thickness of remineralized layer showed, that it is 3.1±0.4 µm for samples treated with SrFCDHAp model toothpaste and 2.0±0.3 µm in case of samples treated with CDHAp model toothpaste.

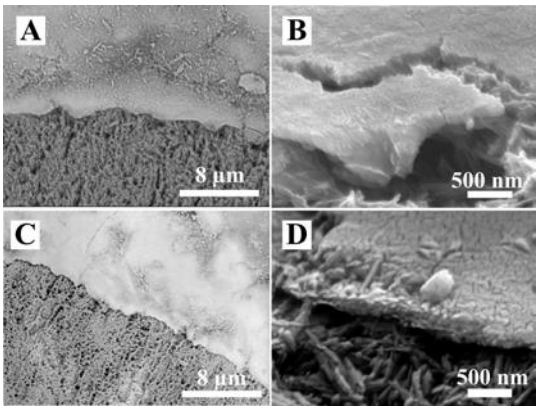

Fig. 1: SEM images: A and B – enamel treated with SrFCDHAp model toothpaste, C and E - enamel treated with CDHAp model toothpaste

DISCUSSION & CONCLUSIONS: Current research was devoted to the global issue – caries prevention. The results of experiment approved, that Sr and F co-substituted CDHAp could be a powerful compound to delay demineralization or promote remineralization of tooth enamel, thus improving the quality of life and and reducing the costs of health care.

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