Towards the communication between nuclei and mitochondria in a hydrogel environment

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INTRODUCTION: Dysfunctional mitochondria play an important role in both neurological and cancer diseases. When this condition arises, the mitochondria send a retrograde signal back to the cell nucleus, which potentially will alter the gene expression and lead to a progression of the disease [1-2]. This process is called mitochondria retrograde signalling. However, our knowledge about this pathway, the molecules involved, and its downstream effects remains limited due to the complexity of simultaneously occurring signalling processes inside mammalian cells. In other words, a major obstacle in studying the mitochondrial retrograde signalling pathway is the missing ability to separate this pathway from the other metabolic activities in the cytosol of a living cell.

Bottom-up synthetic biology is a promising method to create minimal models. Previously, it was demonstrated that purified nuclei and mitochondria could be encapsulated into alginate and gelatin methacrylate, respectively, to create RNA- or energy-producing artificial cells [3-4].

Therefore, we aim to create a minimal cell that can be used as an in vitro platform for investigating mitochondria retrograde signalling without interference from the complex network inside the mammalian cells (fig. 1).

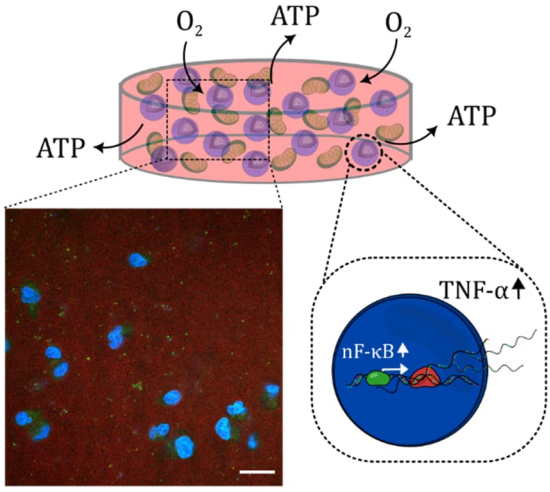

Fig. 1: Minimal cell containing purified nuclei and mitochondria to study relevant markers related to mitochondria retrograde signalling.

METHODS: The minimal cell model was created by purifying nuclei and mitochondria from HeLa and HepG2 cells and encapsulating them into hydrogel networks**.** The retention of structure and functionality of these organelles outside their host cells were evaluated, both in bulk solution and encapsulated into a PEG-based hydrogel. For the nuclei, we examined the mRNA production and the retention of a stimulated inflammatory marker (TNF-α) in the purified nuclei by RT-qPCR. Meanwhile, the core properties examined for mitochondria were ATP production and oxygen consumption.

RESULTS: Mitochondria and nuclei were purified from the donor cells and a successful purification and encapsulation into PEGDA-based hydrogel of the two organelles was confirmed by identification of mitochondria- and nuclei-specific markers with western blot and confocal laser scanning microscopy. Furthermore, the purified mitochondria demonstrated ATP production up to at least one week after purification in bulk solution and encapsulated into hydrogel discs. For the nuclei, mRNA production was demonstrated up to 4 hours after purification. Further, it was confirmed via RT-qPCR, that expression of the cytokine TNF-α could be induced in the donor cells and retained in purified nuclei in bulk and after encapsulation into PEGDA hydrogel discs. This demonstrated that the model could be used to study the expression of relevant markers related to mitochondrial retrograde signalling. Lastly, the organelles were co-encapsulated into a PEGDA-based hydrogel network to produce the minimal cell model.

DISCUSSION & CONCLUSIONS: Mitochondria and nuclei were successfully purified with retained structure and functionality outside the host cells and encapsulated into PEG-based hydrogel discs. Taken together, these efforts represent the initial steps towards in vitro minimal cells that can be used to explore the pathways involved in mitochondria retrograde signaling without having the interference of the complex network inside the mammalian cells.

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ACKNOWLEDGEMENTS The Human Frontiers in Science is acknowledged for funding.

Surface-Modified Nanocellulose Hydrogels for Modulating Macrophage Phenotype in 3D Culture

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INTRODUCTION: The ability of macrophages to switch between proinflammatory (M1) and anti-inflammatory (M2) states determines immune responses to immunotherapies and biomaterials [1,2]. However, conventional 2D *in vitro* culture models provide limited insight into the regulation of macrophage phenotypes. To study macrophage behavior in a 3D tissue-like environment, hydrogels that mimic physical properties of extracellular matrix (ECM) are needed. Nanofibrillar cellulose (NFC) gels are particularly attractive materials due to their sustainability, biocompatibility, and tunable surface chemistry [3]. In this study, we explore NFC gels with different surface chemistries as 3D culture scaffolds to induce M2 macrophage polarization *in vitro* (*Fig. 1*).

METHODS: NFCs derived from birch pulp were used in the study. The physicochemical properties of the chemically modified NFCs (oxidized, phosphorylated, or sulfated) were compared to native NFC with no chemical modifications. Human blood-derived monocytes were cultured inside all NFC gels for up to 6 days, after which their differentiation into M2 macrophages was evaluated using flow cytometry and ELISA. As a control, monocytes were also cultured in a conventional 2D setting on tissue culture plastic.

RESULTS: Chemically modified NFC gels had several advantages over the native NFC gel, including increased transparency. While all NFC gels were negatively charged and had similar mechanical properties, the specific functional group influenced immune cell responses. Among the studied gels, phosphorylated NFC was most efficient in promoting M2-like macrophage generation from monocytes.

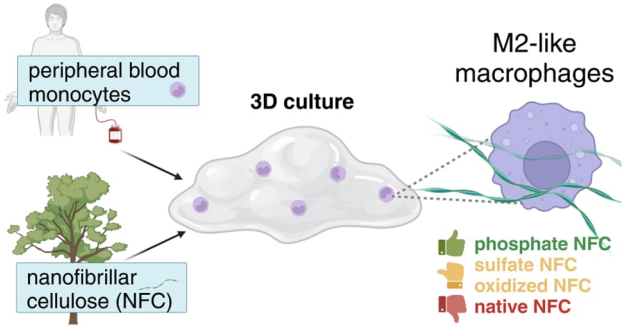

Fig. 1. Schematic overview of the study.

DISCUSSION & CONCLUSIONS: NFC gels enable immune cell culture in a defined 3D environment, where macrophage phenotype can be modulated simply by changing the surface chemistry of NFCs. This approach eliminates the need for costly cytokines typically required in 2D macrophage cultures. Ultimately, using 3D NFC gels to study macrophage phenotypes may contribute to the discovery of new immunomodulatory strategies.

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ACKNOWLEDGEMENTS: This work was supported by grants from Academy of Finland (318890, 318891, 346108, 357914), Finnish Foundation for Technology Promotion, Business Finland (42533/31/2020), Cancer Foundation Finland, Sigrid Juselius Foundation, Finnish Cancer Institute, European Union (847912), and the U.S. Department of Defense (W81XWH2110773).

Digital Light Processing (DLP) Bioprinting of Soft Tissues

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INTRODUCTION: Digital Light Processing (DLP) is a 3D printing technology that uses a digital micromirror device to project UV light patterns onto a resin, curing it layer by layer with high precision. Unlike extrusion-based bioprinting, which relies on mechanical nozzle deposition and suffers from slower speeds and lower resolution, DLP enables superior resolution, faster printing speeds, higher throughput and improved cell viability due to reduced shear stress. This makes it ideal for creating intricate tissue structures.

We employed DLP Bioprinting to fabricate two types of soft tissues: Meniscal tissue [1] and corneal stroma equivalents (CSE) [2]. There is a clinical need for both these tissues. Meniscal tears are the most common sports injuries, with meniscectomies and meniscal allograft transplantation (MAT) being the gold standard of treatment. Meniscectomies lead to osteo-arthritis in the long-term and MAT has supply issues and degeneration over time that requires corrective surgeries. On the other hand, corneal blindness, a leading cause of visual impairment globally, has created a pressing need for alternatives to corneal transplantation due to the severe shortage of donor tissues.

METHODS: Gelatin from porcine skin (CAS: 9000-70-8) and methacrylic anhydride (MAA; CAS: 760-93-0), and carboxymethyl cellulose (CAS: 9004-32-4) were obtained from Sigma-Aldrich (Darmstadt, Germany). GelMA was synthesized using standard procedures with minimal modifications. For the meniscal tissue printing, a 15wt% of GelMA was used (with 0.25wt% LAP), with post-printing crosslinking in tannic acid (35% wt solution). For CSE printing, we formulated a novel interpenetrating network hydrogel composed of GelMA and oxidized carboxymethyl cellulose (OxiCMC).

The formulated hydrogels and bioinks were extensive characterized using FTIR, NMR, uniaxial compression testing, and rheometer for their material, mechanical, and rheological properties.

STL files were modelled using SolidWorks and DLP printing was done using LumenX+ DLP printer.

RESULTS: By using a dual polymerization system with a biocompatible hydrogel, we were able to optimize and fabricate a meniscal scaffold with superior mechanical properties closely matching the native tissue. The dual crosslinking using TA was further verified by the alteration in the structural, morphological, thermal, and physical properties of GelMA. By using DLP printing, we were able to create anatomically accurate scaffolds seeded with hMSCs that further contribute to tissue regeneration.

DLP-printed CSE highlights the novel combination of GelMA and OxiCMC forming a robust hydrogel with enhanced mechanical properties while maintaining optical transparency (>80%), crucial for corneal applications. The dual crosslinking method, involving both Schiff base reactions and photocrosslinking, ensures the hydrogel's strength and stability, making it suitable for 3D bioprinting.

DISCUSSION & CONCLUSIONS: For the meniscal scaffold printing, compared to GelMA, the GelMA-TA scaffold displayed significant antibacterial and antioxidant properties, significantly improved the proliferation of hMSCs, and preserved the chondrogenic differentiation ability of hMSCs. Additionally, GelMA-TA effectively mitigates pro-inflammatory reactions in monocytes while fostering an anti-inflammatory M2a macrophage phenotype, underscoring its potential as an immunomodulatory biomaterial. Incorporating OxiCMC not only improved the mechanical properties but also bioprinted structures showed excellent fidelity to the designed models, demonstrating the hydrogel's suitability for creating complex, anisotropic, and curved shapes like the corneal stroma. Furthermore, biological assessments indicated high cell viability (>93%) and proliferation within the bioprinted constructs at day 7.

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ACKNOWLEDGEMENTS: Do not forget to give credit to those who helped you perform the experiments or funded your research.

Effects of S53P4 Bioactive Glass Extract and Nanofibrillated Cellulose on 3D Bioprinted Human Bone Marrow Stem/Stromal Cells

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INTRODUCTION: In biomedicine, tissue models that mimic better native tissues are highly needed for studying tissue development, diseases and novel therapies. Bone tissue models are needed to study e.g. bone defects and fractures, osteoporosis and cancer metastasis in bone.

Cellulose is an unbranched, linear, polysaccharide that can be isolated from different plants including wood. It is biocompatible, biodegradable and has low cytotoxicity. [1] Furthermore, nanocellulose has been shown to improve bioinks [2] meaning that using this material could solve biomedical engineering problems related to cell culture scaffolds while also reducing the waste caused by the wood industry. Bioactive glass (BAG) has excellent biocompatibility, ability to bind to bone and stimulatory effect on bone cell functions. It has been used as bone-substitute material for nonload-bearing applications in orthopedic and dental surgery.

Here, our aim was to study the effects of BAG and nanocellulose on 3D bioprinted human bone marrow stem/stromal cells (BMSC) and evaluate technique's suitability for bone tissue modeling.

METHODS: Extrusion based 3D bioprinting was used to create 3D bone tissue models. Human BMSC were bioprinted within modified hyaluronic acid bioink supplemented with human collagen type I and nanofibrillar cellulose. The samples were cultured in osteogenic medium with or without S53P4 BAG extract consisting of sodium, silicate, calcium and phosphate ions. Functionality and viability of the cells were studied with Live/Dead assay, immune- and cytochemical staining, the cell proliferation was quantified by using CyQUANT Cell Proliferation Assay Kit and osteogenic differentiation was studied by quantifying alkaline phosphate (ALP) activity. Furthermore, the mechanical properties of the samples were studied with compression testing and micro-CT was used to study the minerals formed during cultivation.

RESULTS: Human BMSC remained viable and obtained elongated morphology (Fig. 2) after 7 days of cultivation indicating good cell attachment in all modified hydrogel samples. Cells expressed osteogenic markers like osteocalcin and collagen I and showed ALP activity. In addition, there was a difference in the ALP results obtained from the samples cultured with or without BAG extract indicating that the BAG extract affects cellular behavior. Furthermore, nanocellulose affected to the printing and mechanical properties of the modified hydrogel.

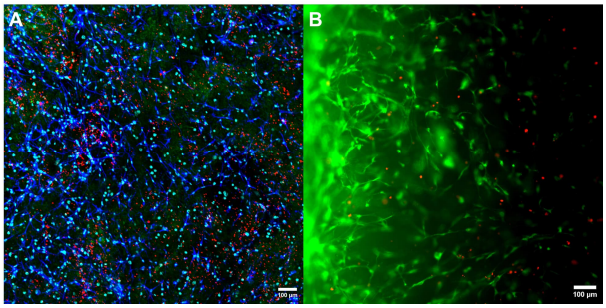

Fig. 2: A. Maximum intensity projection of immuno- and cytochemical stained sample (blue: phalloidin, cyan: DAPI, green: collagen I, red: osteocalcin) and B. Live/Dead stained sample (green: live, red: dead) after 14 days of cultivation. Scale bar 100µm.

DISCUSSION & CONCLUSIONS: Novel hydrogel combined with extrusion-based 3D bioprinting was used to create viable constructs that are able to differentiate towards bone. The role of added nanocellulose and BAG, especially due to its effects on ALP activity, should be studied further for bone applications.

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ACKNOWLEDGEMENTS: Circular Material Network (CIMANET) Doctoral Education Pilot program, Centre of Excellence in Body-on-Chip (CoEBoC) research consortium, Tampere University Research Centre of Science Mimicking Life, Biocenter Finland and Tampere Imaging Facility, Celltech laboratories of Tampere University, Adult Stem Cell research group, technical assistant Anna-Maija Honkala and Sari Kalliokoski

Impact of protein adsorption on bioactive glasses and biological response: a step forward towards a predictable cell fate

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INTRODUCTION: The impact of musculoskeletal diseases is increasing worldwide because of the continuous population aging. This phenomenon is negatively influencing the patients' wellbeing and graving over the societal healthcare system [1]. While a large number of biomaterials have being developed to support musculoskeletal regeneration, a strong discrepancy between *in vitro* and *in vivo* results was observed [2]. This issue was mainly attributed to a lack of understanding of the protein-material interaction, as protein adsorption is known to mediate the interactions between the implant with the surrounding environment. Protein adsorption, indeed, is a crucial phenomenon occurring at the very early stage after biomaterials implantation, determining the cell fate and, therefore, the success of the implant [3].

METHODS: Protein adsorption on bioactive glasses (BGs), biomaterials proven to be clinically safe and efficient in treating musculoskeletal diseases, has been investigated. A screening on four different glass compositions, five surface modifications and three model proteins was performed. Proteins were adsorbed in static and dynamic condition, in order to better mimic the *in vivo* environment. Protein affinity with BGs was evaluated in terms of quantity, surface distribution and conformation, as a function of BG physicochemical properties (Fig 1). The impact of protein adsorption on the biological response was evaluated by studying the viability, proliferation and morphology of human adipose stem cells (hASCs), and the expression of relevant osteogenic markers.

RESULTS: Surface modification impacted on BG physicochemical properties (chemical composition, morphology, wettability, charge). While untreated bioactive glass surfaces exhibit limited protein adsorption, a controlled surface treatment can favour the adhesion of selected proteins. In dynamic, protein adsorption is less efficient, however similar surface distribution, as in static conditions, was reported. When adsorbed, fibronectin changed its conformation towards a more packed structure. All the surfaces showed a good cell viability, proliferation and osteogenesis. Pre-adsorbing fibronectin promoted cell alignment.

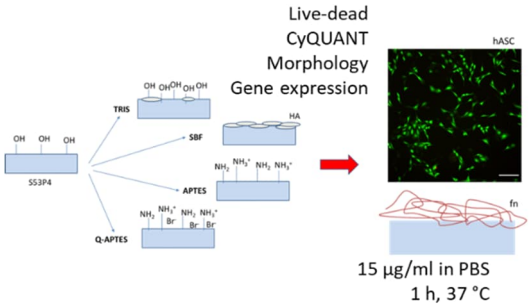

Fig. 1: Summary of the applied methods.

DISCUSSION & CONCLUSIONS: Controlling the surface chemistry and topography enables better prediction of the protein-material interaction as well as controlling which protein is more likely to interact with the materials' surface. The flow of the protein solution plays an important role in the dynamic protein adsorption. Fibronectin adsorption at the glass surface, prior to cell testing with hASCs, primarily affect cell orientation, indicating that proteins at the material surface highly affects the cell behavior, crucial aspect that should be taken into consideration when testing the materials *in vitro*.

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ACKNOWLEDGEMENTS: This study has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860462 (PREMUROSA) and from Tampere University Doctoral School (Tampere, Finland).

Exploring Complementary Anticancer Effect of Pulsed Electromagnetic Field Stimulation on Osteosarcoma *In Vitro* Using 3D-Printed β-TCP Scaffolds

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INTRODUCTION: Pulsed electromagnetic field (PEMF) stimulation is widely employed to promote bone tissue regeneration [1]. Beyond regenerative applications, PEMF has been reported to enhance the efficacy of combination cancer therapies [2]. However, the precise mechanisms by which PEMF influences osteogenesis and cancer cell susceptibility to therapeutics remain elusive. This study investigates the potential of PEMF to sensitize osteosarcoma cells to doxorubicin using a co-culture *in vitro* model developed on a 3D-printed β-tricalcium phosphate (β-TCP) scaffold that has been demonstrated as a suitable bone-like microenvironment for osteosarcoma modelling in our previous work.

METHODS: The scaffolds were fabricated via 3D printing a β-TCP and Pluronic® blend, followed by sintering, and subsequently seeded with primary bone marrow-derived mesenchymal stem cells (pBMSCs). The cells proliferated with the deposition of the extracellular matrix, creating a favourable environment for the subsequent co-culturing of osteosarcoma cell spheroids on the pBMSCs-seeded β-TCP scaffolds. To explore the complementary anticancer potential of PEMF, the developed bi-culture scaffolds were subjected to a PEMF stimulus (1.5 mT, 75 Hz) followed by doxorubicin treatment. The efficacy of the treatment with and without PEMF exposure was analysed by fluorescence microscopy, RT-qPCR, and nanoindentation.

RESULTS: While our earlier scaffold-free spheroid studies showed no significant PEMF-mediated enhancement of osteosarcoma sensitivity to doxorubicin, this study extends the hypothesis to a more advanced co-culture model. By evaluating spheroid morphology, doxorubicin accumulation, osteosarcoma marker expression, mechanosensitive gene profiles, and spheroid stiffness within the bi-culture scaffolds, we assessed the role of PEMF in modulating drug response. Figure 1 demonstrates the bi-culture osteosarcoma model on the β-TCP scaffold used in the study.

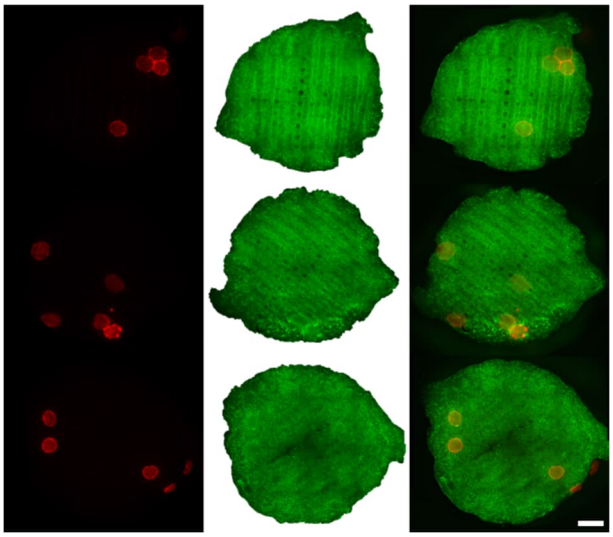

Fig. 1: 3D-printed β-TCP scaffold with bi-culture consisting of osteosarcoma cell spheroids (red) and pBMSCs (green). Scale bar 1000 μm.

DISCUSSION & CONCLUSIONS: Previously, we demonstrated that the co-culture model developed using 3D-printed β-TCP scaffolds effectively mimics osteosarcoma *in vitro*. In the present work, we applied the model to evaluate the potential of PEMF in enhancing the sensitivity of tumorigenic cells to the anticancer drug. We analysed the expression of key osteosarcoma markers and mechanoreceptor-related genes in the co-culture model along with the morphology and stiffness of the spheroids. Our findings, together with the data obtained for the scaffold-free spheroid model, offer new insights into the role of PEMF in anticancer clinical applications.

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ACKNOWLEDGEMENTS: The authors are grateful to IGEA Clinical Biophysics for providing the PEMF stimulator adopted in this study. This research was funded by the European Union's Horizon 2020 Research and Innovation Programme (GA 860462).

Metabolomics-Inspired Biomaterials: Amorphous Calcium Phosphate with Targeted Metabolic Enhancement for Bone Repair

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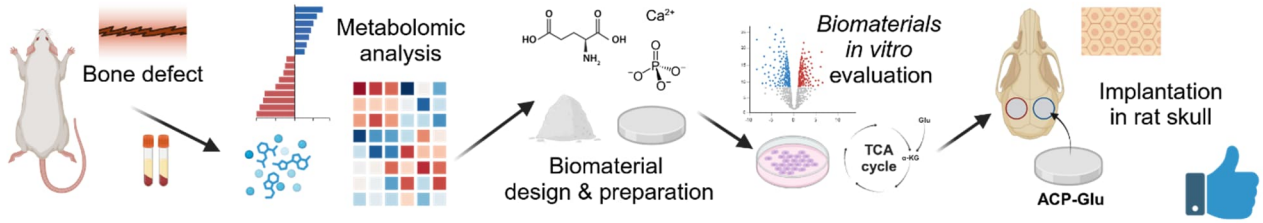

Fig. 1: Schematic diagram of the study, which includes exploring metabolic changes during bone repair, design biomaterials based on animal informatics, and conduct in vitro and in vivo experiments on biomaterials.

INTRODUCTION: In bone tissue engineering, they can mimic the structural and functional properties of natural bone, aiming to promote tissue regeneration. The comprehending the impact of implantable materials on healing processes is pivotal. Metabolomics is the comprehensive study of small molecules (metabolites) within a biological system, providing insights into metabolic processes and their regulation. Our study employed animal models to identify critical metabolites in bone healing and explored the development of Amorphous Calcium Phosphate (ACP) infused with these metabolites to facilitate bone recovery.

METHODS: We used rat and sheep bone defect models to identify systemic metabolic changes during bone healing. Metabolites identified through bioinformatics analysis were used in cell experiments to evaluate mineralization capacity. Amorphous Calcium Phosphate (ACP) and ACP with glutamate (ACP-G) were synthesized from calcium glutamate and characterized using XRD and SEM. These materials, alongside hydroxyapatite and a blank control, were assessed *in vitro* to assess osteogenesis, metabolic analysis, ALP activity, and the expression of osteocalcin and osteopontin. Cold sintered ACP-G were implanted into rat calvaria with critical defect, along with ACP and Bio-Oss® to compare. *In vivo* results were evaluated through tissue section staining and CT scanning.

RESULTS: Metabolomic analysis of serum from animal models with critical bone defects showed decreased glutamine and glutamate levels during bone regeneration. Glutamate and glutamine supplementation enhanced the mineralization capacity of MC3T3-E1 pre-osteoblasts. ACP-Glu, synthesized from calcium glutamate and trisodium phosphate, and the material was amorphous with ~40 nm particles. Glutamate release in culture media followed a linear trend over 3 days. Osteoblasts cultured on ACP-Glu exhibited higher osteopontin and osteocalcin levels, along with improved mineralization (Alizarin Red staining), compared to ACP. Metabolite analysis revealed significant changes in energy metabolism, particularly in the TCA cycle. In vivo, ACP-Glu demonstrated superior bone regeneration compared to traditional biomaterials, highlighting its potential for bone repair.

DISCUSSION & CONCLUSIONS: Metabolomics profiling of rat calvaria and sheep tibia revealed systemic metabolic changes during bone regeneration, particularly the significant downregulation of glutamate and glutamine. ACP-Glu demonstrated exceptional efficacy in enhancing cellular energy metabolism and osteogenic properties. The enhanced energy metabolism, especially the TCA cycle, could compensate for anaerobic glycolysis and provide the energy needed for tissue repair processes. The methodology of biomaterial design and evaluation, inspired by animal model data analysis, and the application of metabolomics aspects, is of great practical value in biomaterials engineering.

ACKNOWLEDGEMENTS: This project has received funding from the European Union's Horizon 2020 research and innovation programme under the grant agreement No. 857287 (BBCE – Baltic Biomaterials Centre of Excellence).

Comparison of Vancomycin-Loaded Injectable Resorbable Phosphate Cements and 3D-Printable Scaffolds for Bone Regeneration and Infection Prevention

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INTRODUCTION: All surgeries carry the risk of complications due to bacterial infections. Infected hip or knee revisions are generally accompanied by local and systemic antibiotic therapy. Generally, in two-stage revisions, non-degradable poly(methyl methacrylate) spacers loaded with antibiotics (ATBs) are used, however, reports show that only 3 - 5 % of ATBs are released *in vivo*[1]. To overcome this issue, we have developed a biodegradable, "self-setting", thixotropic and osteoconductive polymer/calcium phosphate bone cement[2]. In the presented work, we loaded the cement with vancomycin and studied different conditions affecting its release and antimicrobial efficiency *in vitro*, followed by an *in vivo* animal study. For the comparative study, we used the same cement paste loaded with vancomycin for the extrusion 3D printing of bone scaffolds, evaluated both *in vitro* and *in vivo*.

METHODS: The cement consists of tricalcium phosphate powder mixed with a thermosensitive biodegradable copolymer according to Vojtova et al.[2]. The cement was loaded with vancomycin (0.05 - 10 hm%) during mixing to study their release kinetics at physiological conditions by high-performance liquid chromatography (HPLC) compared with ultraviolet-visible spectroscopy (UV-Vis). The same cement pastes were also used for the extrusion 3D printing of porous bone scaffolds. Antibacterial properties were evaluated by disk diffusion method against *Staphylococcus aureus* (SA), methicillin-resistant SA (MRSA), and *E-Coli*. The cytocompatibility of bone cement was evaluated *in vitro* using human mesenchymal stem cells, and the biocompatibility and osteointegration were studied on Wistar rats by filling an iatrogenic bone defect in the femoral bone.

RESULTS: The *in vivo* study shows that the cement was set at physiological conditions with no signals of chronic inflammation. In contrast, slower cell growth was observed *in vitro* compared to control on plastic. Vancomycin-loaded cements were very effective against the tested G+ bacterial strains, even at very low concentrations (0.05%). The antimicrobial capacity was associated with the biodegradation of the cement.

DISCUSSION & CONCLUSIONS: The thixotropic bone cement is an excellent excipient for the controlled first-order release of antibiotics for preventing or healing musculoskeletal infections. The release is strongly dependent on the vancomycin concentration, and the size of the sample and release conditions. The maximum concentration of vancomycin is released within the first 24 hours from the injectable cement, while 21 days of controlled release was observed from the 3D-printed scaffolds. Via HPLC, active and two non-active (minor and major) vancomycin compounds[3] were detected, whereas UV-Vis can detect only the total released vancomycin. The total amount of released vancomycin was comparable via both HPLC and UV-Vis methods. The evaluation of the bone infection treatment by novel antibacterial vancomycin-loaded cement on rat model with SA-infected femoral bones is in progress.

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ACKNOWLEDGEMENTS: This work was supported by the Ministry of Health of the Czech Republic under project no. NW24-05-00202.

3D microrheology for hydrogel microstructure analysis

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INTRODUCTION: Spatial mapping of hydrogel microstructure facilitates discoveries on formation of complex biomaterials and cell-hydrogel interactions. Microrheology is a technique for analysing hydrogel viscoelasticity and porosity at microscopic level using tracer particles1. Passive microrheology with multiple particle tracking (MPT) enables hydrogel composition characterization in high spatial resolution and three dimensions without the need for specialized equipment. In MPT, a hydrogel is filled with fluorescence particles and imaged as time series, from which we can track Brownian motion of the particles. These motion patterns reveal rigid, viscoelastic and fluid-like areas in the hydrogel2. When a specific location is imaged in following days, we can analyse whether or not biodegradation by cells, enzymes, or hydrolysis has happened at the specific location, and if cells have affected hydrogel composition in other ways, gaining essential knowledge to be used in e.g. tissue engineering.

METHODS: We used Nikon Eclipse Ti2 wide field fluorescence microscope, enabling both 2D and 3D imaging, to image hydrogels filled with 200 nm diameter fluorescent particles (Dragon Green, Bangs Laboratories). In our proof-of-concept research, we used Geltrex® hydrogel at 10 mg/mL concentration, where WI-38 human fibroblasts were cultured in 3D. Recordings were done at 40 frames per second with Z levels varied between 1 - 5 µm for a total recorded volume of 595 000 – 925 000 µm^3.

For data analysis and visualization, we developed **MuRheo** software with Unreal Engine 5. MuRheo uses traditional methods[3] for particle detection, and tracking is based on optimized nearest neighbor algorithm. Several parameters can be adjusted to handle various datasets. When 3D data is used, different Z levels are processed in parallel for faster computations. Hydrogel composition visualization is based on Voronoi diagram based on mean squared displacement (MSD) of particles[2]. In addition, movement of single particles can be further studied with built-in plotting tools.

RESULTS: Brownian motion of tracer particles reveals hydrogel microstructure, as presented in Figure 1. By computing amounts of similarly moving particles, we get an overview of hydrogel structure. From 3D mapping we can detect areas where particles move more freely, viscoelastic areas, and rigid areas, defined by MSD and adjustable MSD thresholds. We can also see that tracer particles get attached to cell surface potentially revealing cell locations and enabling analysis of cell-hydrogel interactions.

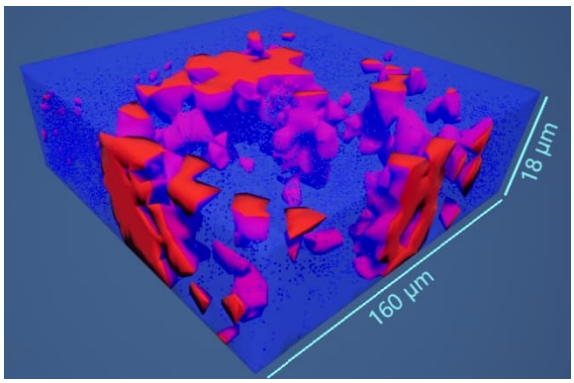

Fig. 1: 3D representation of hydrogel microstructure showing pores in red. Small spheres represent fluorescent particles.

DISCUSSION & CONCLUSIONS: The presented approach for 3D microrheology analysis can be used for various hydrogels and combined with other rheological measurements for comprehensive analysis. MuRheo software is freely available, which we hope to encourage novel experiments with 3D microrheology.

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Relationship between rheological properties and biological activity of porcine skin derived-hydrogels for 3D cell culture

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INTRODUCTION: In their native environment, cells are surrounded by and interact with the extracellular matrix (ECM).[1] However, most biological investigations rely on 2D cell culture on plastic substrate lacking physiological relevance. Switching to 3D cell culture remains a challenge, both technically and in terms of materials to be used. Decellularized extracellular matrix (dECM) is a promising candidate in that respect.[2] Gels derived from dECM possess biochemical cues to assure an adequate cell viability by retaining multiple important components of ECM. We have developed hydrogels with tunable rheological properties and fast gelation from porcine skin, by optimizing the decellularization process and varying the concentration of dECM. We explore here how cell viability, proliferation and spreading are affected by the formulation of these porcine skin-derived hydrogels.

METHODS: dECM was obtained from decellularization of porcine skin tissue, and the presence of the major ECM components and remaining DNA was measured with biochemical assays. dECM pre-gels were prepared by a 24 h-enzymatic digestion of dECM at the concentration of 5 mg.mL^{-1}, followed by pH adjustment to 7.2−7.4 and UV sterilization. Pre-gels were diluted to a concentration of 2.5 and 1 mg.mL^{-1} with sterile PBS. Cell viability and proliferation were monitored over a week with fibroblasts seeded on top of pre-casted hydrogels or encapsulated within the hydrogels. Cell proliferation was assessed with AlamarBlue assay and cell density and morphology observed by confocal imaging after immunostaining.

RESULTS: The decellularization removed 88 % of the DNA while maintaining the levels of collagen, elastin and glycosaminoglycans in the dECM (Fig. 1a), which is comparable with literature.[3] When cultured on top of dECM gels, cells start to spread within 72 h of culture for softer gels (G' < 1 kPa), but only after one week of culture for stiffer gels (G' > 1 kPa). Moreover, cells penetrated the 3D environment of softer gels (Fig. 1b), not that of stiff gels. In addition, fibroblasts could proliferate when encapsulated in the softer hydrogels.

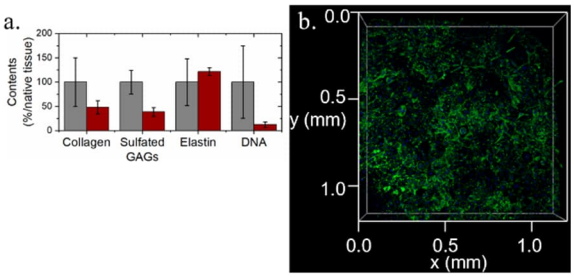

Fig. 1: a. Maintenance of ECM components and DNA after decellularization. b. L929 fibroblasts on top of 2.5 mg.mL^{-1}dECM gel after 1 week of culture.

DISCUSSION & CONCLUSIONS: In this work, we investigated the biological activity of porcine-skin derived hydrogels with tunable rheological properties. When increasing the concentration of dECM in the gel, stiffer gels are obtained. While cells can spread on top of the stiffer gels, they cannot penetrate the 3D environment. However, softer gels allowed for spreading and penetration within the 3D environment by the cells when seeded on top or encapsulated. Our work shows that by tuning the concentration of porcine skin-derived hydrogels, we can formulate substrates for 2D or 3D cell culture. These findings are promising for the wider use of porcine skin-derived hydrogels as bioinks in tissue engineering.

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ACKNOWLEDGEMENTS: We thank August Claesson and Karl Vilhem Ståhl for their contribution to experiments. Carl Tryggers Stiftelse is acknowledged for funding support (CTS 22:2367). This work is conducted within the Additive Manufacturing for the Life Sciences Competence Center (AM4Life). The authors gratefully acknowledge financial support from Sweden's Innovation Agency VINNOVA (Grant no: 2019-00029).

Application of proteomics in the biological characterization of Ca-enriched Ti surfaces: *in vitro* - *in vivo* correlation

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INTRODUCTION: Numerous studies highlight the potential role of calcium ions in the osseointegration of dental implants. The incorporation of Ca^{2+} on Ti surfaces promotes osteoblast adhesion and proliferation by enhancing intercellular communication via receptor activation and cell interaction on osteoblast membranes [1]. Ca^{2+} play a key role in initiating early healing processes, underscoring their significance in the initial phase of bone regeneration [2]. Despite promising *in vitro* results when evaluating new surface modifications, these findings often fail to translate effectively into *in vivo* outcomes [3]. This discrepancy emphasizes the need to evaluate new methods to accelerate the clinical application of innovative regenerative materials. Consequently, developing predictive *in vitro* models is essential to improve efficiency in biomaterial development. In this work, we used proteomics to analyze the effects of Ca-modified Ti surfaces in the surface protein adsorption, *in vitro* responses to HOb and THP-1 cells and the tissue protein expression surrounding modified implants *in vivo*, examining their correlation.

METHODS: Ti surfaces were enriched with $CaCl_2$ and analized by SEM/EDX. Their osteogenic and inflammatory responses were assessed *in vitro* using human osteoblasts (HOb) and THP-1 cells. In addition, implant–blood contact experiments were performed to evaluate the coagulator potential of the Ca-enriched surface. Surface protein adsorption on the Ca-surfaces was evaluated using proteomics, after incubating samples with human serum (2, 180, and 960 min). In parallel, HOb and THP-1 cells cultured on the samples were also eluted and analysed by nLC-MS/MS. Finally, *in vivo* experiments were conducted using a rabbit condyle model. Tissue obtained from implants tested *in vivo* were also analyzed using proteomics.

RESULTS: $CaCl_2$ covered completely the Ti surface forming a homogeneous layer. Ca-modified surface improved HOb growing and increased ALP after 14 days. THP-1 macrophages displayed higher TNF-α levels with Ca samples, which was regulated with the Ca released. Ca-treated implants showed a thick blood clot attached to the whole implant contour with respect to the untreated Ti. Regarding proteomic results, the study of protein adsorption patterns on the Ca-samples showed that this surface modification resulted in increased adsorption of proteins associated with coagulation (FA10, THRB and ANT3) and immune response. However, the presence of immune proteins attached on the samples significantly decreased with the incubation time. In the *in vitro* proteomic analyses, HOb cells on Ca-modified surfaces showed enriched functions in migration, adhesion, ECM organization, and proliferation, involving PI3K-Akt and mTOR pathways. Macrophages initially upregulated proteins linked to adhesion, polarization, and calcium channel activity on Ca-surfaces. However, the proteins related to these effects were found to be downregulated after 3 days. After 5 days of implantation *in vivo*, tissue around Ca-modified implants showed distinct protein profiles, with changes in adhesion, immune response, and bone healing proteins.

DISCUSSION & CONCLUSIONS: Ca-modified surfaces influenced the tissue–material interface by modulating protein deposition and cell responses. Proteomics showed consistent profiles across protein adsorption, *in vitro*, and *in vivo* stages. Unique profiles on Ca-surfaces likely explain their osteogenic, coagulative, and immunomodulatory effects, emphasizing their considerable potential to improve dental implant osseointegration. Overall, proteomics is a valuable tool for predicting biomaterial responses *in vivo.*

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ACKNOWLEDGEMENTS: The authors thank the financial support of PID2023-150429OB-I00 /MCIU/AEI/10.13039/501100011033/FEDER,UE and Generalitat Valenciana through CIPROM/2023/62.

Dual Crosslinking Strategy to Modulate Temporal Oxidation and Enhance Printability in Photocrosslinkable Gallol-Based Biomaterial Inks

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INTRODUCTION: Gallol-mediated crosslinking has attracted considerable interest in biomaterial research due to its ability to form robust hydrogels suitable for various biomedical applications, including bioprinting [1]. However, the auto-oxidation reaction of gallol groups can be challenging to control, leading to suboptimal print fidelity and compromised long-term structural stability [2]. To overcome these limitations, we investigated a dual crosslinking strategy that couples gallol-mediated oxidation with photocrosslinking, aiming to improve temporal control over oxidation, enhance printability, and maintain shape fidelity.

METHODS: Hyaluronic acid methacrylate (HAMA) was selected as the primary polymer backbone for its biocompatibility and tunable mechanical properties; gallol groups were grafted onto HAMA (HAMA-GA) to enable oxidative crosslinking and hydrogen bonding (Figure 1A); pH elevation initiated intramolecular interactions among gallol groups, forming a weak hydrogel network with a temporal auto-oxidation process and optimal shear-thinning behavior for injectability and extrusion; once the pH reached approximately 5.5, ultraviolet (UV) irradiation triggered methacrylate-based photocrosslinking, halting gallol-mediated oxidation while preserving unoxidized gallol groups and stabilizing hydrogen bonds; and an interpenetrating network (IPN) hydrogel was formed, thereby enhancing structural integrity over extended periods.

RESULTS: We found that at pH 5.5, the HAMA-GA biomaterial inks exhibited optimal shear-thinning behavior and printability, whereas higher pH levels led to rapid gelation unsuitable for bioprinting. Controlled auto-oxidation at this pH enabled effective dynamic covalent crosslinking, maintaining the fluidity necessary for extrusion while providing adequate initial structural integrity (Figure 1B). After printing, photocrosslinking significantly enhanced the mechanical stability and adhesive strength of the scaffolds by quenching the oxidation process and reducing brittleness. This dual crosslinking strategy effectively combined oxidative and photocrosslinking mechanisms, improving both cohesive and adhesive properties and resulting in stable, bioprintable hydrogels..

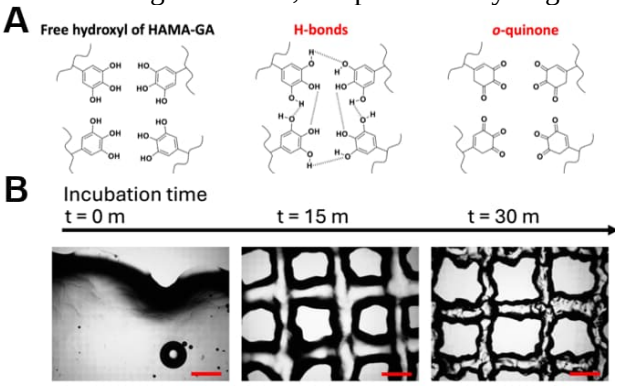

Fig. 1: (A) Effect of pH variation (3.5–8) on the HAMA-GA biomaterial ink precursor, highlighting how temporal oxidation influences the printability window at pH 5.5. (B) The time-dependent printability window was determined by printing HAMA-GA (pH 5.5) into two-layered grid structures. Scale bar = 1 mm.

DISCUSSION & CONCLUSIONS: Our dual crosslinking approach addresses the primary challenges of gallol-mediated auto-oxidation—uncontrolled crosslink progression and limited print fidelity—by coupling pH-triggered oxidative gelation with photocrosslinking to achieve temporally regulated gelation and optimal shear-thinning. The subsequent UV-induced crosslinking halts further oxidation, creating a stable interpenetrating network with long-term structural integrity. This strategy offers a versatile platform for developing biomaterial inks with tailored mechanics, injectability, and extended stability, paving the way for optimizing gallol density, tuning pH triggers, and exploring other photocurable polymers in future tissue engineering and regenerative medicine applications.

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RGD-alginate microbeads as scaffolds for structuring human pulmonary fibroblasts

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INTRODUCTION:

An *in vitro* lung alveoli model with pulmonary fibroblasts can be useful for investigating small-cell lung cancer. Hydrogel microparticles have been suggested as structure-forming elements in granular hydrogels [1]. Alginate, a polysaccharide derived from seaweed, can form microbeads by dripping into a divalent ion solution. Here, we investigated the ability of RGD-grafted alginate microbeads to support the adhesion of primary human pulmonary fibroblasts (HPFa). We studied the effect of two adhesion peptides; linear GRGDSP (linRGD) and cyclic RGD-(D-Phe)-K (cycRGD).

METHODS:

High MW alginate (68 % G, 237 kDa) (UPLVG, NovaMatrix, Norway) was oxidized to 8% before grafting with GRGDSP or RGD-(D-Phe)-K peptide (Caslo, Denmark) with reductive amination [2]. A 4.8% and 7.2% degree of substitution was characterized by ^{1}H-NMR, for linRGD and cycRGD, respectively. Alginate (1.8 % (w/V)) was used to form beads with an electrostatic droplet generator and a gelling solution of 50 mM $CaCl_2$ and 1 mM $BaCl_2$. HPFa were seeded on top of a bilayer of microbeads in static culture at a concentration of 1.3 mM RGD, and cultured at 5% O_2 and 37 ºC. On day 3, the cells were fixed and stained for nuclei (DAPI), cytoskeleton (Phalloidin), and immunolabeled with ki-67 monoclonal antibody, before being visualized by confocal scanning microscopy (CLSM).

RESULTS:

HPFa adhered to the cycRGD-alginate microbeads and spread on the bead surfaces, exhibiting a stretched morphology with cells interconnecting with cells on neighboring beads after 3 days of culturing (Fig. 1). A large proportion of the cells showed proliferative activity through the expression of the ki-67 marker. In contrast, HPFa formed clusters when seeded on linRGD-alginate microbeads. However, when seeding HPFa on flat linRGD-alginate gels, the cells showed increased spreading with higher gel stiffness, indicating that HPFa responds to linRGD as an adhesion ligand. To achieve successful adhesion and spreading on linRGD-alginate beads, additional Mn^{2+} ions in cell culture media were required alongside the linRGD adhesion ligand. Under these conditions, HPFa adhered to the beads and exhibited a stretched morphology after 3 days of culturing (not shown).

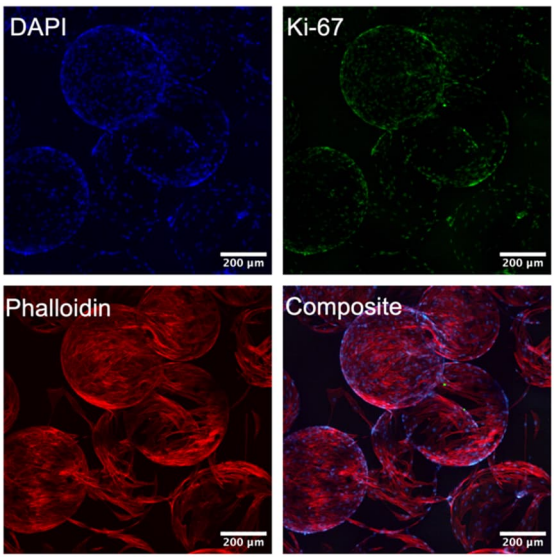

Fig. 1: HPFa were fixed and labeled with DAPI (blue), ki-67 antibodies (green), and Phalloidin (red) after 3 days of culturing with 1.3 mM cycRGD microbeads.

DISCUSSION & CONCLUSIONS:

Alginate is an excellent material for the formation of regular microparticles due to the formation of spherical hydrogels by dripping into a solution of divalent ions. Here, linRGD and cycRGD alginate were used in the formation of beads that allowed the adhesion of HPFa and cellular interconnections between cells on different beads. Hence, both cycRGD- and linRGD-alginate microbeads are promising materials for structuring HPFa. For HPFa on linRGD-alginate gels, increased spreading on linRGD-alginate gels was shown with increased gel stiffness, and Mn^{2+} in culture media was required for adhesion to beads.

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ACKNOWLEDGEMENTS: We are grateful for funding from NTNU Health and The Royal Norwegian Society of Sciences and Letters.

Functionalized Mesoporous Silica Nanoparticles with Redox-Responsive Gatekeeper for Carboplatin Delivery to treat Advanced Ovarian Cancer

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INTRODUCTION: Ovarian cancer (OC) is the leading cause of death among gynaecological cancers, often diagnosed at advanced stages due to non-specific symptoms and limited screening methods. The standard treatment involves cytoreductive surgery followed by platinum-based chemotherapy, such as carboplatin (CARB). However, CARB's efficacy is often compromised by systemic toxicity and poor targeting of tumour cells[1]. Nanoparticle-based drug delivery systems (DDS) offer a promising strategy to enhance therapeutic outcomes by targeting diseased tissues and minimizing side effects. Mesoporous silica nanoparticles (MCM-41) are of interest as drug nanocarriers due to their high surface area, tuneable porosity, adjustable particle and pore sizes, biocompatibility and low toxicity. Functionalizing their surface with molecular gatekeepers allows precise control of drug release in response to specific stimuli[2].

In this study, MCM-41 were loaded with CARB and functionalized with a redox-responsive gatekeeper to control drug release in response to reducing agents like glutathione (GSH), present at high concentrations in the tumour tissue microenvironment [3].

METHODS: MCM-41 were synthesized using cetyltrimethylammonium bromide (CTAB) as a structure-directing agent and tetraethyl orthosilicate (TEOS) as a silica precursor. The nanoparticles were functionalized with thiol groups (MCM-41-SH), loaded with CARB, and sealed with polyethylene glycol (PEG) chains containing disulfide groups (MCM-41-SS-CARB), acting as a redox-responsive gatekeeper for controlled drug release.

The synthesis, functionalization, and drug loading processes were confirmed through various characterization techniques, including transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and thermogravimetric analysis (TGA).

Finally, to qualitatively confirm the gating capabilities of MCM-41-SS-CARB, drug release assays were conducted using GSH as a reducing agent to trigger the release of CARB from the nanoparticles, with the amount released quantified by UV/Vis spectrophotometry.

RESULTS: The successful synthesis, functionalization, and CARB loading in MCM-41 were confirmed through TEM, FTIR, XRD, and TGA. In the release studies, before GSH addition, the absorbance intensity of CARB remained stable, indicating no leakage from the mesopores. Upon GSH introduction, a significant increase in absorbance was observed, demonstrating the redox-triggered release of the drug. The reduction of disulfide bonds in the PEG gatekeeper allowed the nanoparticle pores to reopen, facilitating controlled drug release in response to the reducing agent.

DISCUSSION & CONCLUSIONS: The successful redox-triggered release of CARB from MCM-41 functionalized with PEG-based gatekeepers demonstrates their potential as controlled and targeted DDS. This approach could enhance therapeutic efficacy and reduce side effects by responding to the tumour microenvironment.

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ACKNOWLEDGEMENTS: This research was funded by Instituto de Salud Carlos III, Ministerio de Economía y Competitividad, co-funded by the ERDF European Regional Development Fund (PI20/00115), Direcció General d'Investigació and Conselleria d'Investigació, Govern Balear (FPI/FPI/004/2021 and FPI/FPI/090/2022) and the Annual Plan for the Promotion of Sustainable Tourism for the period 2023 (ITS2023-086 - Programa de Foment a la Recerca) and funded through the Operational Program of the European Regional Development Fund (ERDF).

Chitosan nanoparticles as a vector for co-transfection of anti-fibrotic microRNAs to treat oral submucous fibrosis

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INTRODUCTION: Oral submucous fibrosis (OSF) is recognized as a precancerous disease characterized by excessive extracellular matrixes (ECM) deposition [1]. The active compound arecoline activates the transforming growth factor-beta (TGF-β)/Smads signaling pathway to induce myofibroblast differentiation [2]. Subsequently, it switches on downstream genes related to fibrotic changes and ECM production. Dysregulation of microRNAs (miRs) is involved in the progression of OSF, and miRs manipulation could be a promising therapeutic approach [3]. In addition, paired miRs can cooperatively regulate a target mRNA in a cooperative manner. However, exogenous miRs can be degraded shortly *in vivo*. Otherwise, chitosan, which also possesses an anti-fibrotic capacity, would be an ideal vehicle for miR transfection. Accordingly, the therapeutic potential of co-transfection of anti-fibrotic miRs by chitosan nanoparticles (NPs) was evaluated as a treatment for OSF.

METHODS: The miR/chitosan NPs were fabricated using the ionic gelation method and characterized. Human oral submucosal fibroblasts were subjected to arecoline stimulation to induce myofibroblast differentiation. The arecoline-stimulated OSF fibroblasts were then co-transfected with miRs using chitosan NPs. Cell uptake was demonstrated, the fibrotic genes were analyzed, and the behaviors of transfected cells were evaluated.

RESULTS: For chitosan NPs loaded with miR, the particle size ranged 136.15±32.86 nm with a polydispersity index of 0.15±0.03 and a zeta potential of +17.8±1.3 mV. Transfection efficiency was 38.2%. In addition to downregulating *collagen type III alpha 1* (*COL3A1*), *matrix metalloproteinase-1* (*MMP-1*), *MMP-7*, *tissue inhibitor of metalloproteinase-1* (*TIMP-1*), *TIMP-2* mRNA expressions, co-transfection with multiple miRs resulted in a more significant downregulation of *TGF-β*, *alpha-smooth muscle actin* (*α-SMA*), *COL1A1*, *COL5A1*, and *MMP-3* levels. A Western blot analysis revealed that the miRs co-transfection decreased pSmad2/3, TGF-β, α-SMA, and type 1 collagen protein products. Compared to lipotransfection, miRs co-transfection using chitosan NPs inhibited wound closure cell motility, cell migration, and collagen gel contraction more efficiently.

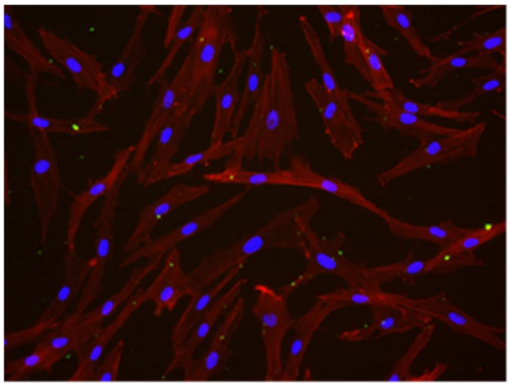

Fig. 1: OSF fibroblasts were transfected with FITC-labeled miRs using chitosan NPs.

DISCUSSION & CONCLUSIONS: Chitosan NP is a good vector for miR transfection. Nanoencapsulation can shield negatively charged groups of miR to feasible cellular uptake. Transfection of anti-fibrotic miRs by chitosan NPs can be a promising treatment for OSF.

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ACKNOWLEDGEMENTS: This study is supported by the Ministry of Science and Technology (MOST 111-2314-B-038-092-MY3).

Liposomal Encapsulation of Cannabidiol: Effects on Properties and Biocompatibility

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INTRODUCTION: Cannabidiol (CBD) boasts remarkable therapeutic properties, but its effectiveness is significantly hampered by poor water solubility and stability, limiting its bioavailability. Encapsulating of CBD in drug delivery systems could enhance its stability and enable more controlled, predictable release. To date, limited research has been conducted on CBD-containing liposomes, though preliminary studies indicate minimal side effects while reducing pain and improving well-being [1]. Current study aims to develop CBD liposomes with varied compositions, assessing CBD concentration and liposome structure on *in vitro* CBD release and cell viability for potential oral disease treatments. Mainly focusing on biocompatibility with gingiva-derived mesenchymal stem cells (GMSC) isolated from patients (Riga Stradins University Research Ethics Committee approval No. 6-1/12/47 (26.11.2020)).

METHODS: Liposome samples were prepared via the thin-film hydration method, varying compositions of commercially available lipids distearoyl phosphatidylcholine (DSPC) and dipalmitoyl phosphatidylcholine (DPPC), and 1,2 distearoyl-sn-glycero - 3 phosphoethanolamine-N-[carbonyl-amino(polyethylene glycol)-4300] (ammonium salt) (DSPE-PEG). Liposomes were characterized using FT-IR (Nicolet IS50) and their size was measured via DLS (Anton Paar Litesizer 500), morphology was analyzed using STEM (Verios 5 UC) with samples prepared on copper grids and stained with ammonium molybdate. CBD release was analyzed using UPLC (Acquity UPLC H-class, Waters). Biocompatibility of liposomes was evaluated on GMSC up to 96h using CCK-8 assay. Further cell differentiation studies were evaluated for osteogenic, adipogenic, and chondrogenic potential.

RESULTS: Liposome sizes, measured by DLS, increased from 657 ± 57 nm (DSPC-DSPE-PEG) to 1167 ± 83 nm (DSPC), with no significant difference between DPPC and DSPC-DPPC, while CBD incorporation reduced sizes by 23-53% across all formulations without altering the size trend. STEM images revealed liposomes with uniform, smooth, homogeneous surfaces and spherical shapes (Fig.1). The highest encapsulation efficiency of CBD was achieved with DSPC-DSPE-PEG CBD at 87%. The release profiles of CBD from liposomes revealed an initial burst release followed by sustained CBD levels releasing up to 83% of encapsulated CBD over 21 days. The cell viability of GMSCs treated with CBD liposomes varied by composition, concentration, and time, DSPC CBD liposomes maintained GMSC viability above 70% (non-cytotoxic per ISO 10993-5:2009), while other formulations (DPPC CBD, DSPC-DPPC CBD, DSPC-DSPE-PEG CBD) exhibited cytotoxicity at specific time points: 72 h, 24-48 h, and 24 h, respectively. The highest GMSC viability was observed after 96 h, showing no cytotoxic effect for all CBD liposome formulations.

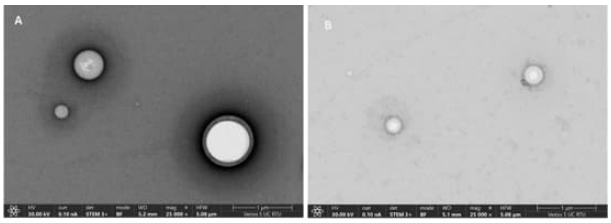

Fig. 1: STEM images: (A) DSPC liposomes, (B) DSPC liposomes with incorporated CBD.

DISCUSSION & CONCLUSIONS: CBD was successfully incorporated into different compositions of liposomes. The encapsulation of CBD into the liposomes led to more compact and potentially more stable particles. Encapsulating CBD in liposomes improves the bioavailability of CBD by protecting it from immediate breakdown.

REFERENCES: [1]L.Y Shilo-Benjamini et al. (2023) *J Vet Pharmacol Ther* **46** (S1):73–73.

ACKNOWLEDGEMENTS: This work was supported by the M-era.Net 2 project INJECT-BIO, No. ES RTD/2020/14, EU Horizon 2020 project No. 857287 BBCE and postdoctoral grant No. RTU-PG-2024/1-0011, EU RRF project No 5.2.1.1.i.0/2/24/I/CFLA/003.