Optimised cartilage construct of preliminary assessment

BioTrib deliverable D3.7 report

WP3 Additive manufacturing

Lead beneficiary: Uppsala University
Reported by: Marie Moulin (ESR 6)
Dissemination level: Public
Due date: PM36
Contributors: Michael Kontakis, Marie Moulin, Brittmarie Andersson, Norein Norein, Ayan Samanta, Christina Stelzl, Adam Engberg, Anna Diez-Escudero, Cecilia Persson, Johan Kreuger, Nils Hailer.

Project title: [Advanced Research Training for the Biotribology of Natural and Artificial Joints in the 21st Century]
Project Number: [956004]
Project Acronym: [BioTrib]

The BioTrib ETN project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 956004.

Table of content

I. Abstract

Cartilage tissue exhibits low self-repair capacities due to its non-vascularized nature and low cell density limiting the production of new tissue in pathological conditions such as osteoarthritis. Despite the development of several clinical strategies to repair cartilage, the treatment of cartilage damage remains challenging. Tissue engineering strategies involving scaffolding biomaterials and cells aim at creating physiologically relevant tissue constructs for *in vitro* tissue models as well as clinical strategies for tissue replacement [1] [2]. Bioprinting technology has made significant progress in arranging biomaterials and cells in specific configurations to create a 3D scaffold in a layer by layer fashion. The objective of 3D bioprinting is to facilitate 3D cell culture and mimic tissue structures, leading to the development of more accurate and physiologically relevant tissue constructs. One major limitation to bioprint functional tissue constructs is the creation of stable complex structures with soft hydrogel like collagen which represent the main extracellular matrix (ECM) component of cartilage. The innovative 3D bioprinting technique known as freeform reversible embedding of suspended hydrogels (FRESH) overcomes this limitation and was applied in this study to form complex porous structures adapted for 3D cell culture [3] [4]. The technique was adapted for use with an open-source extrusion bioprinter, and a collagen bioink to create a biomimetic microenvironment for cell growth. The printing parameters were optimized to create the intended porous geometry with collagen, providing the constructs with specific mechanical and biological properties. The bioprinted porous scaffolds proved compatible with cell adhesion, survival and infiltration during one month of culture, as monitored by confocal microscopy and cell viability assessment. However, the collagen-based scaffolds exhibited limited resistance to mechanical forces, and future efforts will therefore be needed to enhance the mechanical properties of the construct for cartilage tissue engineering. Moreover, the biological properties of the constructs will have to be further investigated with advanced characterization method quantifying gene expression (qPCR) and protein production (Western Blot) using a clinically relevant cartilage cell line. The current study is the result of the FRESH printing process optimization to produce collagen constructs with complex porous geometry and its preliminary assessment for potential application in cartilage tissue engineering.

II. Introduction

The construction of relevant tissue models for studying cells in healthy and pathological contexts are important for deepening our understanding of how cell interactions with their surroundings impact their responses to signaling molecules and pharmacological agents. Furthermore, the capacity of such models to recapitulate physiologically relevant 3D architecture and mechanical properties of particular tissues like cartilage, increases their utility as translational model systems, with potential future clinical applications. 3D *in vitro* models provide a more biomimetic approach than traditional 2D models, and also represent ethically preferable alternatives to pre-clinical animal testing, and finally have the potential to facilitate clinical translation of tissue-engineering solutions [5]. For example, synovial joints may be affected by different pathological conditions such as osteoarthritis. Hence, osteoarthritis damage the cartilage and underlying bone resulting in pain and functional disability for the patient [6]. Different tissue engineering approaches are under investigation to create constructs mimicking the properties of cartilage and evaluate their potential for *in vitro* tissue models [2] and replacement strategies for damaged cartilage which exhibits low self-repair abilities *in vivo* [1]. The creation of 3D tissue models can be performed through a scaffold-based approach focusing on replicating the various functions of the natural extracellular matrix (ECM) through an engineered 3D

structure which will support cell adhesion and survival [7]. Amongst biofabrication technologies, extrusion-based bioprinting holds great promise to achieve reproducible tissue scaffolds that mimic complex tissue architecture and functionality. Bioprinting enables the precise deposition of a bioink in accordance with a predetermined pattern, most commonly in a layer-by-layer fashion [8]. This automated method of replicating designed patterns brings greater control over reproducibility, scaffold heterogeneity and porosity as compared to other biofabrication techniques of porous scaffolds by freeze drying or solvent casting. Control over porosity is essential for the creation of relevant tissue constructs as it allows for transport of nutrients and metabolic waste to support cell viability over time within the 3D construct [9]. The cells interacting with the printed scaffold, turning it into a "living structure" called construct, can be printed within the bioink or seeded on the scaffold post-printing. Here, the term bioink is generally used to refer to acellular hydrogel-based compositions.

- FRESH bioprinting

Extrusion-based bioprinting facilitate the construction of advanced 3D tissue constructs, in which components of interest from the ECM like collagen can serve as bioinks to create hydrogel scaffolds, which can be used to grow cells and replicate tissue properties of interest. The mechanical extrusion force driving the bioink from the syringe out through the needle is applied either by pneumatic, piston or screw-driven systems and allows to deposit continuous bioink filaments. One of the major limitations when it comes to producing complex scaffolds using ECM proteins like collagen is the ability to bioprint mechanically stable scaffolds, which do not collapse or deform under their own weigh [3] [4]. To address this limitation, several strategies using supportive material [10] have been described like the freeform reversible embedding of suspended hydrogel method (FRESH) developed in 2015 in Feinberg's laboratory [3]. FRESH bioprinting allows for more complex and structurally stable porous scaffold fabrication, by printing soft bioink in a bath whose role is to physically supports the extruded filament without the need for an underlying bioink filament. The support bath is a sacrificial, biocompatible gelatin microparticle slurry which exhibits low mechanical resistance when subject to shear stress created by the extrusion needle path allowing for continuous bioink extrusion in the bath. However, without shear stress, the gelatin microparticle bath is rigid enough to support the extruded hydrogel holding it in a fixed and suspended state. When acidic collagen is printed into a gelatin microparticle bath having a physiological pH, this will lead to pH-driven collagen self-assembly allowing to stabilize the filament. In a post printing step, the scaffold in the gelatin support bath is transferred in a 37 °C incubator, allowing to melt the gelatin to discard it without damaging the scaffold, and to further self-assemble the collagen [4]. The collagen scaffold with physiological pH can then be used for cell seeding and as a long-term 3D cell culture model to study cell behavior.

Collagen represents the main ECM component of cartilage together with proteoglycans which help retaining water within the cartilage ECM to provide its mechanical properties responsible for load transmission. FRESH bioprinting using biologically relevant collagen bioink to create complex porous structure hold great potential for the creation of biomimetic cartilage constructs.

III. Aims

This study aims at creating an optimized cartilage construct and assess its mechanical and biological properties. To create the construct, first, the novel FRESH method will be implemented on an open source bioprinter [11]. Moreover, using this method, a collagen based bioink will be evaluated for its printability in order to create scaffolds with controlled interconnected porosity which are mechanically stable. The constructs will be optimized in term of print fidelity in order to create the

complex intended geometry with pores allowing cell infiltration for prolongated cell culture over time. Once the optimized combination of printing parameters will be defined, the viscoelastic and mechanical properties of the 3D scaffolds will be evaluated alongside with the biological properties of the constructs seeded with cells. Indeed, mechanical properties of the collagen scaffold are influenced by the fabrication process as well as the geometry and porosity. Cell infiltration, survival and proliferation in the constructs will be investigated over a month of culture with fluorescent microscopy and enzymatic assay. These characterizations represent preliminary biological and mechanical evaluation of the engineered 3D constructs after printing parameters optimization. Further biological investigations will have to be performed to evaluate its potential application for cartilage tissue engineering.

IV. Material and methods

a. Scaffold fabrication

The scaffold model was designed using the Netfab software (Autodesk) as a cylindrical 3D structure with a solid periphery and an anisotropic porous configuration. The computer aided design (CAD) file was sliced in the Simplify3D software using the following optimized printing parameters: extrusion needle of 27G, printing speed of 10 mm s^{-1} and layer height of 100 µm.

The collagen scaffolds were printed using the FRESH method implemented on an open source extrusion bioprinter based on the E3D motion system and tool changer [11]. An acidic solution of bovine collagen type I (Lifeink 240 Collagen Bioink, Cellink) at a concentration of 35 mg mL^{-1} was extruded into a neutral pH gelatin support bath (FRESH LifeSupport, Cellink).

After printing the collagen scaffolds were moved into 96-well plates for sterilization by immersion in ethanol for 20 min.

b. Material characterization

- Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and scanning electron microscopy (SEM)

A Fourier Transform Infrared (FTIR) spectrophotometer (Shimadzu IRTracer-100) was used to obtain FTIR spectra of the self-assembled type I collagen resulting from FRESH bioprinting in the range of 400–4000 cm^{-1}.

The microarchitecture of the collagen scaffolds was imaged using a scanning electron microscope (SEM; Zeiss LEO 1550). Collagen scaffolds were dehydrated using a critical point dryer (Polaron E3000) and coated with Au/Pd using a sputter coater (Polaron SC764).

- Viscoelastic properties

Viscoelastic properties of the FRESH printed collagen scaffold were investigated using a rheometer (DHR2, TA instruments). Amplitude sweeps were performed to plot the storage (G') and loss (G") modulus (Pa) against the deformation (strain in %) of the printed scaffolds and identify the linear viscoelastic region. The following parameters were used: constant frequency of 0.1 Hz and torque varying from 0.01 to 1.0 µN.m. A 8 mm geometry was used, which corresponds to the smallest geometry available from TA instruments. As the 3D scaffold diameter is inferior to the geometry size, the corresponding parameter was modified in the rheometer software to obtain a more accurate calculation of the oscillation stress.

- Mechanical properties

Mechanical properties of the FRESH printed collagen scaffolds were assessed by performing an uniaxial unconfined compression test on a rheometer (DHR2, TA instruments) using a 40-mm parallel plate stainless steel geometry. A compression speed of 5000 µm.min^{-1} was used until the scaffolds were deformed by 30% of their initial height. The stress (Pa) was calculated using the sample diameters at the start of the experiment and the compressive Young's modulus was extracted from the slopes of the stress strain curves obtained for four compressed scaffolds.

c. Biological characterization

- Cell source

In this study, a human osteosarcoma cell line was used. Cells were seeded on the collagen scaffolds and in 96-well plates (uncoated). The 2D cell culture condition was used as the control condition. The culture medium (DMEM + 10% FBS + 1.5% antibiotics) was changed twice a week for a total of four weeks. In addition, an osteoblast cell model was also used in this study due to its availability in the collaborator team with whom this study was carried out (Department of surgical Sciences, Uppsala University Hospital) and as a preliminary means of assessing cell adhesion, survival and infiltration into the collagen-interconnected porous scaffold.

- Cell infiltration and viability

Cells infiltrating into the porous scaffold was visualized with confocal laser scanning microscopy using a Carl Zeiss LSM 700 Laser Scanning Microscope (Carl Zeiss) after 1, 2, and 4 weeks of culture. After cell fixation, cell nuclei were stained with DAPI (Invitrogen) and cell cytoplasm with carboxyfluorescein diacetate (CFDA; Merck KGaA). Intracellular osteocalcin (OCN) was detected with immunofluorescence using the anti-OCN antibody (MAB1419; R&D Systems). The obtained Z stacks resulting from the fluorescent imaging were post-processed into maximal intensity projection in Image J.

Cell viability was measured with lactate dehydrogenase (LDH) colorimetric assays at 1, 2, and 4 weeks of culture after cell lysis using an enzymatical lysis buffer. The In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (TOX7, Sigma) was used. Absorbance measurements were performed using a spectrophotometer (Multiscan Ascent, Thermo Fisher Scientific) after 30 min. The absorbance at 690 nm was subtracted from the absorbance at 492 nm, and the resulting values were expressed as fold change in comparison with control values (cells in 2D culture). Two biological replicas were performed, using three technical replicates for each time points.

V. Results

The objective of this study was to bioprint a stable porous structure using ultra-soft collagen type I which could be used for cartilage tissue engineering. Collagen scaffolds were printed using the recently described FRESH bioprinting method [3], and characterized with regard to their mechanical and biological properties. The biological properties were evaluated *in vitro* by seeding osteoblast cells onto the bioprinted collagen scaffolds.

a. Scaffold fabrication

FRESH bioprinting was successfully used to produce porous scaffold featuring overhanging structures and interconnected pores. The concentrated collagen I (35mg mL^{-1}), exhibited good printability with

the following printing parameters: 10 mm.s^{-1}, 210 µm internal diameter needle. Within the Simplify3D software, other specific printing parameters were optimized like “perimeter overlap” and “endpoint extension distance”, to obtain 3D scaffolds with high shape fidelity, meaning that the printed scaffold replicate the original design of the CAD file [12]. This combination had to be optimized to avoid over-extrusion which could lead to limited porosity as well as under-extrusion which could lead to discontinuous printing and loss of integrity. Following an iteration procedure of printing parameters optimization, the FRESH printed collagen I scaffold faithfully replicated the cylindrical model. The collagen scaffolds were stable enough for post printing handling.

b. Material characterization

- FTIR and SEM

Fourier-transform infrared (FTIR) spectroscopy of the self-assembled type I collagen resulting from FRESH bioprinting showed the characteristic bands of the collagen protein: amide I (1641 cm^{-1}), amide II (1547 cm^{-1}), and amide III (1238 cm^{-1}) [13].

Scanning electron microscopy (SEM) was employed to show the microarchitectural properties of the printed collagen. The collagen constructs exhibited a complex intricate and fibrous 3D network of collagen fibrils. It should be noted that sample preparation for SEM included dehydration of the hydrogels, which can impact the microarchitecture of a structure primarily composed of water, potentially introducing artefacts in the observed structures. Nevertheless, these SEM images are a valuable tool to observe the collagenous fibril network.

- Viscoelastic and mechanical properties

Both rheological and mechanical tests were performed on the collagen scaffolds post-printing. Rheology examines the reaction of viscoelastic materials like hydrogels to deformation, as they can exhibit traits resembling that of both purely viscous fluids and elastic solids under varying conditions. Moreover, since the scaffolds evaluated in the present study are preliminary tested for cartilage tissue engineering, it is important to evaluate elasticity and stiffness through mechanical testing.

Subjecting the collagen scaffold to increasing shear stress during an amplitude sweep test, showed that the scaffold exhibited a predominantly elastic behavior over viscous behavior, with a storage modulus G’ being higher than the loss modulus G”. G’ and G” were determined within the linear viscoelastic region. Even though the collagen scaffold behaved like a viscoelastic solid, its resistance to compression was low with a compressive Young’s modulus greatly inferior to reported values for human articular cartilage [14].

c. Biological characterization

The presence and distribution of osteoblasts in the collagen scaffolds after seeding and extended periods of culture were assessed by confocal microscopy, and the cells infiltrated the porous scaffold. As the human cell line used in this study was osteoblastic, the maintenance of their phenotype was confirmed by osteocalcin (OCN) immunostaining, a well-established osteoblast marker.

The viability of cells was assessed using an LDH activity assay. Lactate dehydrogenase is an enzyme find in all living cells which catalyze the interconversion of pyruvate and lactate. LDH activity measured on cell lysate is used as a proxy for cell number. Collagen scaffolds maintained cell viability and growth of the seeded cells over four weeks.

VI. Discussion

In this study, FRESH bioprinting workflow was successfully implemented on the in house developed open source 3D bioprinter [11]. FRESH bioprinting was used to create a stable interconnect porous construct suitable for 3D cell culture. Collagen type I was used as biocompatible and biomimetic substrate for cell adhesion, survival and growth mimicking ECM function. The constructs were optimized to produce a scaffold geometry with high shape fidelity to the intended computer-aided design. The appropriate combination of printing parameters allowed for proper printing resolution, creating the intended interconnected porous structure. Even though FRESH bioprinting allow to print stable collagen scaffolds, the printing process optimization is still essential to produce relevant constructs. The porous architecture enabled cell infiltration and offered a suitable platform for 3D cell culture over extended culture time. Indeed, the 3D interconnected porous structure provides a greater specific surface area compared to 2D cell culture. This method, which uses a rapid pH change to direct collagen self-assembly in the gelatin support bath, allows the printing of highly concentrated (35 mg mL^{-1}), chemically unmodified collagen as a bioink, and avoids the use of a cytotoxic chemical cross-linking agent [4]. FRESH allowed to bioprint a complex 3D model with collagen with overhanging features which would not feasible without the function of the support bath.

The mechanical properties of the scaffolds were inferior to those of human articular cartilage due to the inherently soft nature of the collagen hydrogel as compared to synthetic polymers with tailorable mechanical properties [15]. However, collagen represents the ECM main component with physiologically relevant sites for cell adhesion, making it a very relevant and therefore widely investigated polymer for cartilage tissue engineering [16]. Collagen type I was used due to its commercial availability in high concentration for FRESH bioprinting while cartilage is mainly composed of collagen type II, but collagen type I is commonly used as a natural hydrogel in cartilage tissue engineering [16] [17]. For example, bovine collagen type I (2 mg mL^{-1}) was used by Lan et al., in 2020 to bioprint human nasoseptal chondrocytes-laden collagen hydrogel for cartilage tissue engineering using FRESH bioprinting [18].

VII. Conclusion

In conclusion, FRESH bioprinting enables fabrication of interconnected porous scaffolds using collagen I, to provide a suitable 3D microenvironment for *in vitro* cells infiltration and growth over 1 month of study. Future research into the utilization of FRESH in cartilage tissue engineering should include clinically relevant models, including human chondrocyte cell lines C20A4 and C28/I2. Furthermore, the collagen bioink could also be mixed with proteoglycans, to generate hybrid bioink formulations, with tunable mechanical and bioactive properties to mimic cartilage tissue composition and properties while maintaining fine printability and shape fidelity for FRESH bioprinting.

Regarding the characterization of the constructs, it is important to highlight the fact that in 3D bioprinted constructs, mechanical properties are dynamic and evolve over time as cells accelerate construct degradation and also produce new ECM. The rheological and mechanical properties of the constructs after different periods of time of cell culture could be compared, to investigate such effects. In addition, it would be interesting to study the gene expression and matrix production of clinically relevant chondrocyte cell lines, in order to characterize chondrocyte phenotype maintenance and behavior on the FRESH-printed scaffolds.

VIII. References

[1] C. Vinatier and J. Guicheux, "Cartilage tissue engineering: From biomaterials and stem cells to osteoarthritis treatments," *Annals of Physical and Rehabilitation Medicine*, vol. 59, no. 3, pp. 139–144, Jun. 2016, doi: 10.1016/j.rehab.2016.03.002.

[2] H. Dou *et al.*, "Osteoarthritis models: From animals to tissue engineering," *J Tissue Eng*, vol. 14, p. 204173142311725, Jan. 2023, doi: 10.1177/20417314231172584.

[3] T. J. Hinton *et al.*, "Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels," *Sci. Adv.*, vol. 1, no. 9, p. e1500758, Oct. 2015, doi: 10.1126/sciadv.1500758.

[4] A. Lee *et al.*, "3D bioprinting of collagen to rebuild components of the human heart," *Science*, vol. 365, no. 6452, pp. 482–487, Aug. 2019, doi: 10.1126/science.aav9051.

[5] P. K. Chandra, S. Soker, and A. Atala, "Tissue engineering: current status and future perspectives," in *Principles of Tissue Engineering*, Elsevier, 2020, pp. 1–35. doi: 10.1016/B978-0-12-818422-6.00004-6.

[6] N. E. Lane *et al.*, "OARSI-FDA initiative: defining the disease state of osteoarthritis," *Osteoarthritis and Cartilage*, vol. 19, no. 5, pp. 478–482, May 2011, doi: 10.1016/j.joca.2010.09.013.

[7] J. C. Valdoz *et al.*, "The ECM: To Scaffold, or Not to Scaffold, That Is the Question," *IJMS*, vol. 22, no. 23, p. 12690, Nov. 2021, doi: 10.3390/ijms222312690.

[8] R. P. Lanza, R. S. Langer, J. Vacanti, and A. Atala, *Principles of Tissue Engineering*, 5th ed. Cambridge, MA, USA: Academic Press, 2020.

[9] N. Zhu and X. Che, "Biofabrication of Tissue Scaffolds," in *Advances in Biomaterials Science and Biomedical Applications*, R. Pignatello, Ed., InTech, 2013. doi: 10.5772/54125.

[10] S. Ji and M. Guvendiren, "Complex 3D bioprinting methods," *APL Bioengineering*, vol. 5, no. 1, p. 011508, Mar. 2021, doi: 10.1063/5.0034901.

[11] A. Engberg, C. Stelzl, O. Eriksson, P. O'Callaghan, and J. Kreuger, "An open source extrusion bioprinter based on the E3D motion system and tool changer to enable FRESH and multimaterial bioprinting," *Sci Rep*, vol. 11, no. 1, p. 21547, Nov. 2021, doi: 10.1038/s41598-021-00931-1.

[12] A. Schwab, R. Levato, M. D'Este, S. Piluso, D. Eglin, and J. Malda, "Printability and Shape Fidelity of Bioinks in 3D Bioprinting," *Chem. Rev.*, vol. 120, no. 19, pp. 11028–11055, Oct. 2020, doi: 10.1021/acs.chemrev.0c00084.

[13] K. Belbachir, R. Noreen, G. Gouspillou, and C. Petibois, "Collagen types analysis and differentiation by FTIR spectroscopy," *Anal Bioanal Chem*, vol. 395, no. 3, pp. 829–837, Oct. 2009, doi: 10.1007/s00216-009-3019-y.

[14] W. Kabir, C. Di Bella, P. F. M. Choong, and C. D. O'Connell, "Assessment of Native Human Articular Cartilage: A Biomechanical Protocol," *CARTILAGE*, vol. 13, no. 2_suppl, pp. 427S-437S, Dec. 2021, doi: 10.1177/1947603520973240.

[15] N. A. Peppas, J. Z. Hilt, A. Khademhosseini, and R. Langer, "Hydrogels in Biology and Medicine: From Molecular Principles to Bionanotechnology," *Adv. Mater.*, vol. 18, no. 11, pp. 1345–1360, Jun. 2006, doi: 10.1002/adma.200501612.

[16] C. Chung and J. A. Burdick, "Engineering Cartilage Tissue," 2009.

[17] F. Chicatun, G. Griffanti, M. D. McKee, and S. N. Nazhat, "Collagen/chitosan composite scaffolds for bone and cartilage tissue engineering," in *Biomedical Composites*, Elsevier, 2017, pp. 163–198. doi: 10.1016/B978-0-08-100752-5.00008-1.

[18] X. Lan *et al.*, "Bioprinting of human nasoseptal chondrocytes-laden collagen hydrogel for cartilage tissue engineering," *FASEB j.*, vol. 35, no. 3, Mar. 2021, doi: 10.1096/fj.202002081R.