



3D Bioprinting Strategies To Engineer Structurally Organised Meniscal Tissue Grafts

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Doctor of Philosophy

Xavier Barceló Gallostra

Supervisor: Prof. Daniel J. Kelly

Internal Examiner: Prof. Conor T. Buckley

External Examiner: Prof. Chang H. Lee

Declaration

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Xavier Barceló Gallostra

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Summary

Lesions to the meniscus are common sports-related injuries that can lead to articular cartilage degeneration and osteoarthritis if left untreated. While surgical repair procedures and meniscal substitutes offer better clinical outcomes compared to meniscectomies, there are still complications and reoperation risks. Therefore, there is a need for new strategies to repair meniscal tissue, which has motivated the investigation of 3D bioprinting, which aims to develop patient-specific implants that mimic the anatomy, structure, composition, and biomechanics of the native tissue. This thesis focuses on using bioprinting to engineer meniscus tissue analogues with biomimetic collagen organisation and tissue phenotype.

To achieve this goal, the first objective was to develop novel bioinks that can rapidly degrade while maintaining desired shapes post-printing. Alginate blends with partially oxidised alginate (OA) were used, and the rate of degradation was dependent on the OA content. These bioinks supported robust chondrogenic differentiation of mesenchymal stem/stromal cells (MSCs), resulting in the development of hyaline-like tissues rich in type II collagen. The tuneable nature of these bioinks offers numerous benefits from providing space in a controllable manner for new extracellular matrix deposition to alleviating concerns associated with a foreign body response to printed material inks *in vivo*.

Inkjet bioprinting was employed in Chapter 4 to deposit a cell-laden bioink into scaffolds of different architectures, aiming to engineer fibrocartilage grafts with user-defined collagen architectures. Polymeric scaffolds with guiding fibre networks of varying aspect ratios were produced using fused deposition modelling (FDM) or melt electrowriting (MEW). Scaffold architecture influenced the spatial organisation of the collagen network, with higher aspect ratios supporting the formation of structurally anisotropic tissues. MEW scaffolds exhibited mechanical properties similar to the native

meniscus, and the tensile properties could be tuned by altering the aspect ratio. Spatially distinct fibre patterned scaffolds were also achieved, leading to the development of heterogeneous tissues with isotropic or anisotropic collagen networks.

Chapter 5 investigates the impact of temporally removing glycosaminoglycans (GAGs) during tissue development on the collagen network maturation. Temporal depletion of GAGs using chondroitinase ABC (cABC) enhanced collagen fibre diameter without affecting tissue phenotype or extracellular matrix production. Tissues treated with cABC exhibited superior tensile mechanical properties compared to empty MEW scaffolds, highlighting the benefit of enzymatic treatments in engineering anisotropic tissues.

Chapter 6 addresses the challenge of mimicking the complex structure, composition, and biomechanics of the native meniscus. Zone-specific meniscus progenitor cells (MPCs) isolated from the inner and outer regions of the meniscus were used to bioprint fibrocartilaginous tissues. By depositing MPCs within MEW scaffolds with elongated pore shapes, anisotropic tissues resembling native cellular niches were successfully engineered. MPCs generated a more fibrocartilage-like tissue compared to MSCs. Additionally, an iterative MEW process was implemented to print scaffolds with a wedged-shaped profile similar to the native meniscus, enabling the engineering of large tissue constructs rich in sGAG and collagen.

Finally, in Chapter 7, a novel concept of directing spontaneous cell self-organisation through 3D bioprinting is introduced. Alginate-based bioinks were used to deposit cells within specific locations, providing geometrical boundary conditions that guided the growth and internal structure of self-organised tissues. This approach allows for the biofabrication of structurally organised meniscal fibrocartilage.

In conclusion, this thesis presents novel biofabrication frameworks for engineering biomimetic meniscal tissue grafts. MEW scaffolds with tailored architecture can modulate collagen fibre directionality and tensile mechanical properties. Temporal

depletion of GAGs improves collagen network maturation and mechanical properties. Zone-specific MPCs enable the recapitulation of meniscus zone-specific features. Large-volume, wedge-shaped anisotropic fibrocartilaginous tissues can be engineered using the MEW process. Additionally, a bioprinting platform leveraging alginate-based boundary conditions directs cell self-organisation. These findings lay the foundation for regenerative treatments for meniscus repair using tissue engineering approaches.

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Chapter 1: Introduction

1.1. The clinical problem with meniscus repair

The soft tissues within the knee facilitate normal movement and function in the body's largest joint; these include the meniscus, ligaments, tendons, and articular cartilage. Over a 10-year period, 39.8% of sport-related injuries were reported to involve the knee joint, with over 40% of patients aged between 20 and 29 years old [9]. Individuals who sustain a knee injury are 7.4 times more likely to develop knee osteoarthritis than those who do not have a history of knee injury [10]. Specifically, meniscus injuries can contribute to the development of osteoarthritis because damage to these structures alters joint loading. Furthermore, among patients with clinical and radiographic findings of osteoarthritis, meniscal lesions are prevalent in 68 to 90% of cases [11]. Meniscus tears may result in symptomatic and asymptomatic knee joints and can be a result of acute knee trauma or degenerative processes [12]. Although such tears occur in all age groups, the patient's age is known to play a role in the aetiology and pathophysiology of meniscal injuries [13, 14].

The menisci are wedge-shaped fibrocartilaginous structures that are responsible for the maintenance of joint stability, distributing axial loads across the joint and thus protecting the underlying articular cartilage [15, 16]. Current treatment options for meniscal injuries include nonoperative treatment, meniscectomy, repair, and transplantation [17]. Given the association of meniscus deficiency and osteoarthritis, by where damage or loss of the meniscus can contribute to the development or progression of osteoarthritis in the knee joint, there has been an increased interest in meniscus repair and preservation where possible [18-21]. Following meniscectomy, the tibiofemoral contact area decreases by approximately 50%, while the contact forces increase 2- to 3-fold [22, 23]. Therefore, it is not surprising that long-term clinical results have reported poor outcomes following partial and total meniscectomy [24]. Furthermore, advances in

arthroscopic techniques and technologies have made meniscus repair a more amenable procedure for surgeons. Preservation of meniscal tissue and function is paramount for long-term joint survivorship, especially for young patients.

Unfortunately, not all meniscus tears can be repaired, especially if considerable damage has occurred [25]. In appropriate patients, meniscus transplantation offers the potential to restore partial load-bearing meniscus function, decrease symptoms, and provide chondroprotective effects [26]. While the results of this operation vary, studies continue to justify the procedure in young patients who have undergone meniscectomy [27]. The cumulative survivorship of meniscus transplantation greatly varies depending on tissue processing, secondary sterilisation, preservation, and operative technique. Investigation into the implant survival rate show values of 50 to 80% survivorship [28, 29]. Overall, the results of such an operation are more favourable when it is performed before the onset of advanced tibiofemoral joint arthritis, and before alteration in joint geometry occurs [30].

Although allograft transplantation has been shown to delay the progression of joint degeneration and allows the majority of patients to gradually regain the ability to perform daily activities, shortage of donor menisci, challenges with the sizing of allograft meniscus and compatibility concerns are limiting the widespread clinical adoption of this intervention [27, 31, 32]. A number of meniscal scaffolds are also in clinical use, notably the Collagen Meniscus Implant CMI[®] (Stryker) and the polyurethane-based Actifit[®] (Orteq) scaffold, however, these implants do not consistently provide a chondroprotective effect [33-36]. There is, therefore, a distinct clinical need for new strategies for the repair of meniscal tissue, which has motivated the investigation of 3D printing technologies for the development of anatomically accurate, patient-specific implants that mimic the structure, composition, and biomechanics of the native tissue.

1.2. 3D bioprinting as a tool for meniscus tissue engineering

Tissue engineering therapies are appearing as promising regenerative treatments as they offer the potential to repair damaged meniscus by using a combination of biomaterials, cells, and bioactive factors [17, 34, 37, 38]. Although meniscus repair products are not as numerous as articular cartilage products and fewer clinical trials have been performed, preliminary clinical data suggest positive outcomes for cell-based therapies [17]. For example, the ‘Cell Bandage’ is a putative therapy that combines mesenchymal stem/stromal cells with a collagen scaffold to drive the healing of avascular meniscal tears [39]. The cell-seeded scaffold is placed in between the torn edges of the meniscus, and the tissue is reapproximated using sutures. The first human trials (EU Clinical Trials Register, 2010-024162-22) in a cohort of five patients (median age of 37 years) presenting with isolated medial meniscus tears (median length of 30 mm) in the avascular zone showed promising results. The device was considered safe and without major complications associated with the surgical procedure or subsequent patient rehabilitation. At around 15 months post-implantation, two patients underwent partial meniscectomy due to re-tear of the torn region. The three remaining patients showed improvements in different clinical scores up to 24 months with the resolution of symptoms and with MRI scans showing a decreased signal intensity over time, indicative of successful healing [39].

Realising tissue engineering strategies capable of biologically regenerating damaged meniscus will require addressing several challenges, including the development of functional biomaterials which not only provide mechanical support but which are also capable of supporting spatially defined and tissue-specific differentiation of stem/progenitor cells. A central challenge when engineering implants for meniscus regeneration is recapitulating the spatial complexity of the native tissue. The majority of the collagen fibres within the meniscus are circumferentially oriented, allowing the meniscus to resist high tensile forces and function as a transmitter of load within the knee

joint [4, 40]. 3D bioprinting is an emerging technology with the potential to enable the engineering of such biomimetic implants as it enables the spatial patterning of biomaterials, cells, and biological factors. In particular, 3D printing strategies such as fused deposition modelling (FDM) and melt electrowriting (MEW) can enable the fabrication of porous scaffolds which recapitulate aspects of the circumferential collagen arrangement of the native meniscus [3, 8, 41, 42]. Moreover, 3D bioprinting can be integrated with patient-specific data to develop scaled-up, anatomically accurate implants for meniscus regeneration. Advances in 3D bioprinting are already supporting the engineering of meniscal-like constructs, including the development of bioinks to support a meniscal cell phenotype, however bioprinting tissues with an anisotropic collagen architecture that mimics that of the native tissue remains a challenge [43].

1.3. Objectives of the thesis

The overall goal of this thesis is to leverage emerging multiple-tool biofabrication platforms, in combination with tissue-specific progenitor cells derived from the inner and outer regions of the meniscus, to engineer tissues that recapitulate the spatial organisation, composition, and mechanical anisotropy of the native meniscus. To realise this goal, this thesis will address the following specific aims:

Aim 1: To develop a toolbox of bioinks with tuneable degradation rates supportive of chondrogenic differentiation of MSCs. Negative foreign body responses following the *in vivo* implantation of bioprinted implants motivate the development of novel bioinks which can rapidly degrade and support the formation of functional tissue, whilst still maintaining desired shapes post-printing. Here, the oxidation of alginate will be investigated as a means to modify the degradation rate of alginate-based bioinks for (fibro)cartilage tissue engineering applications. These alginate blends will be combined with a temporary viscosity modifier (gelatine) to produce a range of degradable bioinks with rheological properties suitable for extrusion bioprinting. Such tuneable bioinks are

expected to support the chondrogenic differentiation of MSCs, and offer numerous benefits to the field of 3D bioprinting, from providing space in a controllable manner for new extracellular matrix deposition to alleviating concerns associated with a foreign body response to printed material inks *in vivo*.

Aim 2: To develop a multiple-tool biofabrication strategy that enables the engineering of spatially organised fibrocartilage tissue. The meniscus is characterised by an anisotropic collagen fibre network which is integral to its biomechanical functionality. However, the engineering of structurally organised meniscal grafts that mimic the anisotropy of the native tissue remains a significant challenge. Here, inkjet bioprinting will be used to deposit a cell-laden bioink, which was developed in the previous aim, into additively manufactured scaffolds of differing architectures to engineer fibrocartilage grafts with user-defined collagen architectures. Polymeric scaffolds consisting of guiding fibre networks with varying aspect ratios will be fabricated using either FDM or MEW. Scaffold architecture is hypothesised to influence the spatial organisation of the collagen network laid down by the jetted cells. Such bioprinted tissues could potentially form the basis of new treatment options for damaged and diseased meniscal tissue.

Aim 3: Application of enzymatic treatments to improve the organisation and mechanics of 3D bioprinted meniscal tissue. The collagen network within *in vitro* engineered fibrocartilaginous tissues is typically rather immature (e.g., the size of the fibres produced in such systems are small compared to native tissue), resulting in the development of tissues with relatively poor mechanical properties. Enzymatic bioactive regimes have previously been applied to engineered (fibro)cartilage to result in improved tensile properties. Building on the strategy developed in the previous aim, scaffolds with defined pore architectures will be used to enable the bioprinting of anisotropic tissues with collagen fibres preferentially oriented parallel to the long axis of the scaffold pores. The

aim here is to determine if the temporal removal of glycosaminoglycans (GAGs) during the early stages of *in vitro* tissue development within a guiding polymeric framework using chondroitinase ABC (cABC) will have an impact on the composition, structural organisation, and biomechanics of engineered meniscal tissues.

Aim 4: Bioprinting of scaled-up meniscal grafts by spatially patterning phenotypically distinct meniscus progenitor cells within melt electrowritten scaffolds. While developments have been made in the field of meniscus regeneration, the engineering of cell-laden constructs that mimic the complex structure, composition, and biomechanics of the native tissue remains a significant challenge. This can be linked to the use of cells that are not phenotypically representative of the different zones of the meniscus, and an inability to direct the spatial organisation of engineered meniscal tissues. Building on the previous aims, the potential of zone-specific meniscus progenitor cells (MPCs) to generate functional meniscal tissue following their deposition into MEW scaffolds was assessed. First, fibronectin-selected MPCs from the inner and outer regions of the meniscus will be investigated for their ability to maintain their differentiation capacity with prolonged monolayer expansion. Using MPCs isolated from the inner (iMPCs) and outer (oMPCs) zone of the meniscus, this thesis aims to engineer highly organised and phenotypically distinct tissues mimetic of the different zones of the meniscus. An iterative MEW process will be then implemented to print scaffolds with a similar wedged-shaped profile to that of the native meniscus, into which iMPCs and oMPCs will be deposited in a spatially controlled manner. Taken together, it is expected that such convergence of emerging biofabrication platforms with tissue-specific progenitor cells can enable the engineering of complex tissues such as the meniscus.

Aim 5: 3D bioprinting hydrogel boundaries to direct tissue organisation. The different microarchitectures of biological tissues are associated with their unique mechanical

behaviour and functionality. In the case of the meniscus, the tissue functions by distributing the loads throughout its circumferentially oriented collagen fibres. To date, the recapitulation of its complex collagen architecture remains a formidable challenge, which may dramatically limit the translation of tissue engineering strategies to clinical utility. In the previous aims, we have shown the ability to direct cellular self-organisation *in vitro* by providing appropriate physical boundaries resulting in constructs that mimic the anatomical shape, internal structure, and composition of the meniscus. However, the material investigated to provide boundary conditions was polycaprolactone (PCL), which degrades slowly *in vivo* and undergoes plastic deformation and failure when exposed to extensive stresses, motivating the search for alternative strategies. By using the toolbox of bioinks developed in the first aim of the thesis, it is hypothesised that hydrogel boundaries can direct cellular self-organisation in a similar way to PCL. Such an approach would provide control over the structural organisation of the bioprinted construct that eventually can lead to the development of “scaffold-free” engineered tissues.

Chapter 2: Literature Review

2.1. Introduction

The meniscus tissue was originally considered a vestigial structure, but decades of research have begun to unravel how its unique structure and composition allow it to provide critical load-bearing functions within the knee joint [16, 23, 44]. The neonatal meniscus tissue is highly vascularised and cellularised, however, this vascularisation significantly decreases with growth and skeletal maturation, which contributes to the poor regenerative capacity of the adult tissue [45]. High impact sports have been shown to significantly impact the knee, since about 40% of sports-related injuries involve this joint, of which 15% are meniscus injuries [9, 46, 47]. If left untreated, damage in the meniscus can be considered a pre-arthritis condition and can lead to other secondary joint complications due to the loss of its protective function [48]. For that reason, meniscus repairs are gaining popularity, although the type, geometry and location of the injury will determine the outcome of the surgical approach [19]. Even though meniscal repairs provide better clinical outcomes compared to total and partial meniscectomies, there are risks associated with large reoperation rates [49]. Meniscus allografts have shown 70% survivorship 10 years after implantation, however, only in 41% of the cases was there an absence of additional joint space narrowing [50]. To counteract the challenge of the poor self-healing potential, representing a formidable orthopaedic challenge, effective and innovative repair strategies are required.

3D bioprinting has emerged as a promising and attractive approach with numerous studies demonstrating its capacity to engineer spatially complex fibrocartilaginous tissue *in vitro* with the potential to be implanted *in vivo* [51-53]. In this literature review, the unique spatial composition, structure and biomechanics of the meniscus are first described, followed by a summary of the relatively limited success that existing implants have had in regenerating a tissue that replicates the complexity and function of the native

meniscus. Next, the recent advancements in 3D bioprinting for the biofabrication of meniscal substitutes, along with the opportunities and challenges associated with using such emerging technologies, are discussed. Finally, this review will describe how the microarchitecture of engineered tissues can be controlled, potentially enabling the biofabrication of functional meniscal grafts for tissue engineering and regenerative medicine applications.

2.2. The importance of matrix structure and composition to the meniscal function

The C-shaped menisci are located between the femur and tibia and can be broadly divided into the medial and lateral meniscus [54, 55]. Their particular semilunar shape, wedged shaped in cross section, is essential for increasing the contact surface between the relatively planar tibial plateau and the more rounded femoral condyles. Tibial plateau coverage by the meniscus ranges between 50% medially and 58% laterally; this anatomical organisation reduces the stress acting over the articular cartilage [56]. The meniscus is often classified into 2 main zones: (1) the red or outer zone and (2) the white or inner zone (Figure 2.1). The red zone is the thickest in the vertical direction and predominantly composed of type I collagen (80% by dry weight), oriented circumferentially along the periphery of the meniscus tissue [48, 57]. 10-30 % of the red zone is enriched with blood vessels that branch out from the popliteal artery nourishing the oval and fusiform-shaped fibroblast-like cells [58]. On the contrary, the white zone is devoid of blood vessels, thus depending on the synovial fluid diffusion (a mechanism supported by joint loading), and populated with round-shaped fibrochondrocytes or chondrocyte-like cells [59]. Moreover, this zone is comprised of densely crosslinked type II (60% of the total collagen) and type I (40% of the total collagen) collagen fibrils together with large proteoglycans, with chondroitin sulphate as a major glycosaminoglycan constituent [57, 60].

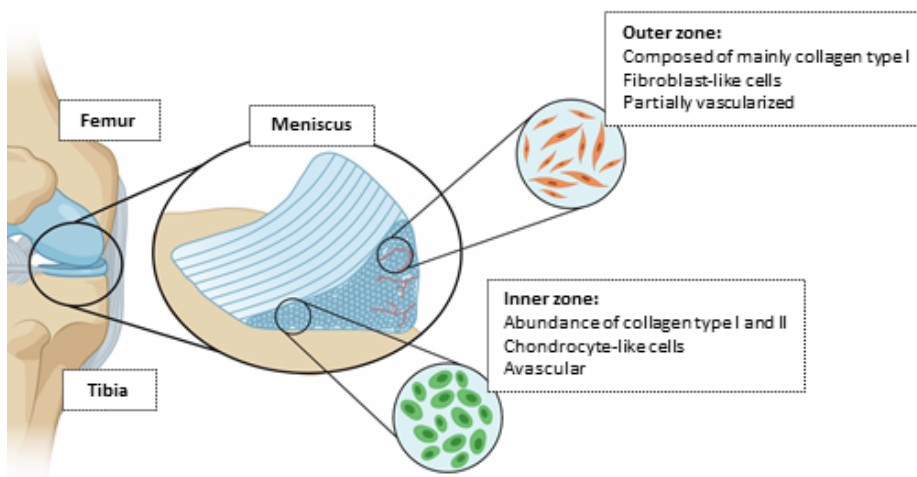


Figure 2.1. Schematic diagram of the knee anatomy and meniscus structure and architecture. The menisci are located between the femoral condyles and the tibial plateau. The menisci contain a highly organised collagen network with fibres mainly oriented circumferentially with few fibres oriented radially. Adult menisci present low levels of vascularisation, which in turn defines a more vascular region, referred to as the outer zone, and a region devoid of blood vessels referred to as the inner zone. These different regions differ in cell and biochemical composition. The outer zone is mainly composed of collagen type I and populated by elongated fibroblast-like cells, whereas the inner zone is composed of collagen type I and type II while presenting more rounded cells, often named chondrocyte-like cells.

The main function of the meniscus is to maintain stability and bear, transfer and distribute the loads while increasing the joint congruency throughout all the range of motion of the knee [16, 44, 61]. The lateral and medial menisci transmit 70% and 50% of the vertical axial forces within the knee joint, respectively, and convert them to hoop stresses throughout the circumferentially arranged collagen fibres [23]. To effectively exert its functionality, the meniscus can partially move during the bending and flexing of the joint, thus ensuring maximum congruency with the articulating surfaces while avoiding injuries to it [62]. The unique collagen microarchitecture defines an anisotropic tissue, with a tensile modulus reaching 100 – 200 MPa in the circumferential direction, and 10-fold lower in the radial direction [44]. Approximately, the human meniscus is composed of water (72%) and collagen (22%), the latter being primary responsible for the tensile strength of the menisci [37, 63]. The meniscus is also viscoelastic, in large part due to the proteoglycans present in the tissue, accounting for 1-2% of the dry weight,

which facilitate hydration [48]. This fluid phase pressurises and flows following the application of load, thereby allowing the tissue to act as a shock absorber within the knee joints with an average aggregate modulus under axial compression of 90 to 160 kPa [64]. Other matrix proteins include fibronectin which is involved in processes such cell adhesion and migration, and tissue repair and regeneration [60].

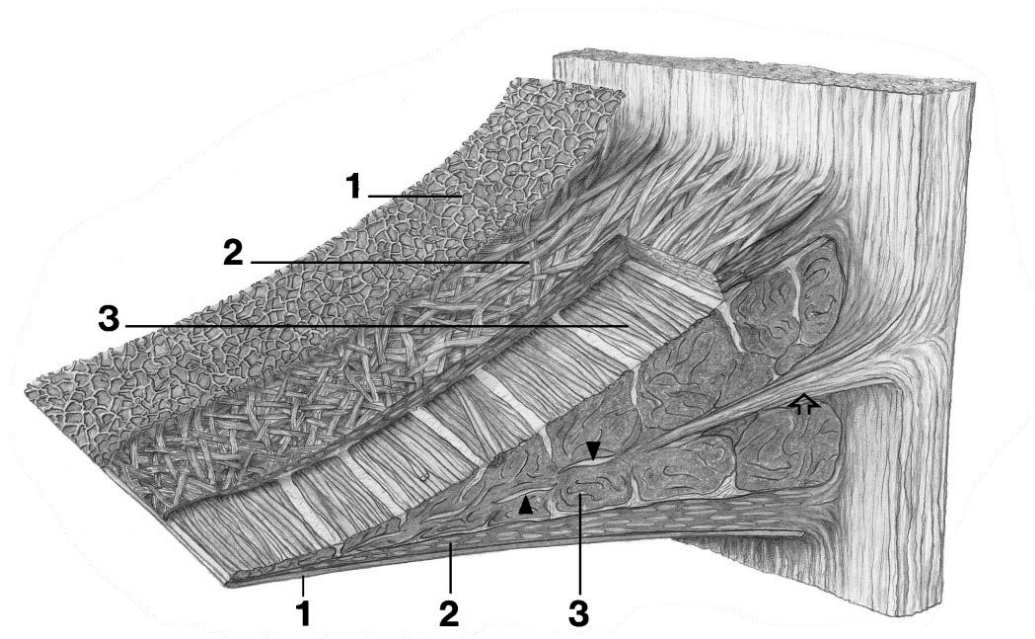


Figure 2.2. Illustration showing collagen fibre ultrastructure and orientation within the meniscus [4]. 1, superficial layer; 2, lamellar layer; 3, central main layer where collagen fibrils have circumferential orientation. Arrowheads, interwoven radial collagen fibrils; open arrow, connective tissue penetrating from joint capsule.

The arrangement of collagen fibrils in the meniscus tissue is ideal for transferring vertical compressive loads into circumferential “hoop” stresses divided into three different organisational patterns (Figure 2.2) [4, 40, 65, 66]. The superficial layer which covers 10 μm from the meniscus surface and is in contact with the femoral and tibial surfaces. It is composed of randomly oriented collagen nanofibrils forming a low friction surface. Beneath the superficial layer, a mesh of about 100-200 μm of collagen fibres extends radially from the external area of the meniscus. Finally, the central and deeper layer of the meniscus tissue possess collagen fibrils bundles that run circumferentially

with few interwoven radial fibres intersecting in between, maintaining the circumferentially oriented collagen bundles together.

In spite of their extraordinary mechanical properties, menisci are prone to lesions, often referred to as meniscal tears, due to the daily extensive loads, with an annual incidence rate of 66 per 100,000 habitants in the United States [67-69]. Interestingly, recreational activities such gymnastics, tennis or jogging account for the highest risk of meniscus injury [9]. Based on the tear pattern, meniscal tears are often classified under the following major types: longitudinal, horizontal, radial, degenerative and bucket-handle tears [48]. Identifying and classifying the injury is essential to choose the correct treatment option as untreated meniscus tears are a potential risk for the development of osteoarthritis. For example, although radial tears may interrupt the functionality of the meniscus by disrupting the circumferentially arranged collagen fibres, the location and depth will determine if they are amenable for repair [13]. Thus outer region tears are most frequently repaired, whereas inner region tears are often excised to alleviate mechanical discomfort [70]. Due to degeneration and declining vascularisation with age, repair has been less successful in older patients, for which tears are commonly of a more complex nature.

2.3. The limitations of current repair strategies

The menisci and articular cartilage of the knee have a close embryological, anatomical and functional relationship, with injury to the meniscus commonly initiating degenerative changes to articular cartilage and other joint tissues and eventually the development of osteoarthritis [71]. This is a global problem. For example, over 1,600,000 meniscectomies and meniscus repair procedures were performed in France between 2005 and 2017, and approximately 35,000 meniscus surgeries are conducted annually in Japan [72, 73]. Repairs of the torn region using anchors and sutures are increasingly common, for which excellent success rates are achieved in young patients [17, 23, 74]. Repair of meniscus tears when conducted in conjunction with reconstruction of a torn anterior cruciate

ligament (ACL) is associated with a 7% decrease in the reoperation rate compared to meniscus repair alone [75]. This has been correlated to the possible release of cells, active factors and bone marrow to the meniscus when drilling bone tunnels which are necessary for ACL reconstruction, providing a rationale to investigate repair through biologic augmentation [70, 76]. Although these treatment options provide adequate repair for young patients, the rate of success in older patients is extremely low for which injuries are generally associated with joint degeneration [20]. Overall, meniscus repair surgeries have a high revision rate, with over 20% of the patients requiring further therapy, and in many cases repairing tears is not possible [49, 77]. Only certain types of tears are amenable for meniscus repair, depending on the size (≤ 4 cm in length), location (red-red or red-white), orientation (longitudinal), and patient age (≤ 40 years old) [25].

Partial or total meniscectomies are therefore still a common treatment option when the tear cannot be healed or successfully repaired by other means [76]. This involves removing the torn section of the meniscus to relieve patients of acute symptoms such as pain or locking of the joint. Removing parts of the tissue does not restore the full function of the meniscus in the long term, and typically leads to further joint degeneration and osteoarthritis [78, 79]. Studies have shown that if only 20% of the meniscus is removed through surgery, the contact forces in the knee are increased by 350% [80]. Under certain circumstances, mainly following a meniscectomy in younger patients, meniscus allograft transplantation is recommended to delay the progression of joint degeneration [26]. After allograft implantation, the majority of patients gradually regain the ability to perform daily activities and practice sport [27]. Allograft transplants are therefore considered by many to be the ideal treatment for treating meniscal deficiency in younger patients, however challenges such as the limited availability of donor menisci and graft size fitting have limited the wider clinical utility of such approaches for meniscal repair [31, 32, 81]. Long-term results after allograft transplantation have shown further joint space narrowing and cartilage degeneration, with a cumulative allograft survivorship of 70% after 10 years

[28, 82]. Therefore, there is a huge clinical demand for alternative treatment options for the repair and regeneration of meniscal tears.

Currently, there are three off-the-shelf products approved by regulatory authorities for clinical use: the Collagen Meniscus Implant CMI[®] (Stryker), polyurethane-based Actifit[®] (Orteq) and polycarbonate-urethane based NUsurface[®] (Active Implants) [35]. The CMI[®] and the Actifit[®] implant replacement procedures follow similar strategies as a partial meniscectomy with the removal of the damaged tissue (Figure 2.3). The crescent-shaped spongy implants can then be resized to replace the damaged tissue and serves as a scaffold for the formation of new meniscus-like tissue [83]. Both implants consist of a homogenous porous mesh and require an intact meniscal rim and horns for correct attachment. Whereas the non-resorbable polycarbonate-urethane (PCU)-based implant NUsurface[®] does not require fixation to bone or soft tissues, but still requires an intact meniscal rim of at least 2 mm in order to fit the disc-shaped device [84]. This device is designed to replace rather than regenerate the damaged tissue.

Mid- and long-term follow-up studies of CMI[®] and Actifit[®] implants have shown improvement compared to meniscectomies in terms of pain relief as well as regaining mobility [33, 83, 85]. However, failure rates of 7 to 30% have been described with

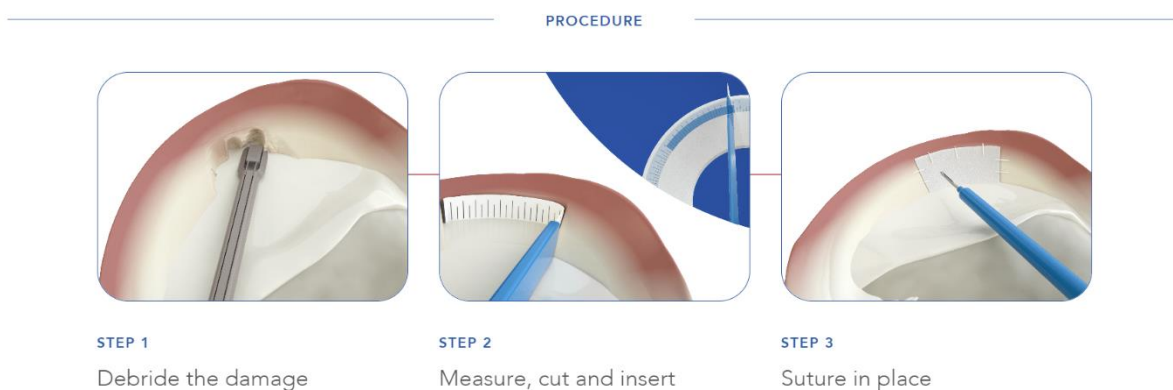


Figure 2.3. Actifit[®] implantation procedure (Orteq[®]). Following the location of the defect and removal of the damaged tissue. The polyurethane meniscus implant is then trimmed with an extra of some mm of the measured defect to compensate for the effect of the horizontal sutures, which partially shrinks the Actifit[®] device.

patients reporting that they were not able to perform sports at the same level as prior to their injury [33, 86]. The non-resorbable implant NUsurface[®] was designed to have the mechanical stability to withstand the loads of everyday movement. However, its inability to be restructured to the shape of the tissue can lead to implant failure or rupture [35]. Thus, there is a distinct clinical need for the development of regenerative implants that can withstand the loads of everyday life without compromising on function and tissue integration.

2.4. 3D Bioprinting for meniscus tissue engineering

Numerous tissue engineering strategies have been explored for the biofabrication of functional meniscus grafts *in vitro* [37, 38]. While these studies have significantly advanced the field, the engineering of anatomically defined meniscal grafts that recapitulate the complex regional composition and structural organisation of the native tissue remains elusive. This has motivated increased interest in the field of 3D bioprinting, which enables the patterning of biomaterials, living cells and bioactive factors arranged systematically into predetermined 3D architectures [87].

2.4.1. Key Components to 3D bioprinting functional meniscal grafts

Bioprinting has the potential to address a number of major challenges in the field of meniscus tissue engineering, in particular the development of anatomically defined grafts with a spatial architecture and composition mimetic of the native tissue. This can be enabled by using non-invasive diagnostic imaging techniques like magnetic resonance imaging (MRI) and computed tomography (CT) scans to generate anatomically accurate representations of the tissue or organ of interest. From this, a working model of the entire tissue, or a specific region of interest, can be generated for 3D printing [42]. This section will explore how different cell types, bioinks and bioregulatory cues can be combined into the bioprinting workflow to engineer functional meniscal tissue.

Cell sources

The meniscus contains distinct zones containing unique and highly specialised cell types [88]. The cell populations can be divided into three distinct groups: (1) small round chondrocyte-like cells located in the inner zone of the meniscus, (2) elongated fibroblast-like cells found in the outer zone of the meniscus, and (3) fusiform cells populating the superficial layers of the meniscus [89]. Cells in the inner and the outer zones of the meniscus tissue display different behaviours in response to joint-specific mechanical stimulation, which emphasises the importance of the distinct meniscus cell phenotypes [90]. The ability of cells to transduce mechanical forces into biochemical signals by promoting anti-catabolic effects attracts increasing attention, hypothesising that the meniscus might be an active contributor to the joint homeostasis [91, 92]. This suggests that the specialised cellular component of the meniscus plays an active role in maintaining a healthy functional knee joint [93]. Therefore, the bioprinting of functional meniscal tissue will either require the use of differentiated, zone-specific meniscal cells, or stem/progenitor cells with the capability to differentiate into such cells if provided with appropriate cues.

Cells harvested from the various zones of the meniscus have been extensively investigated for their histocompatibility and for producing a similar tissue phenotype as found *in vivo* [34]. Moreover, cells isolated from the inner avascular part accumulate more GAGs in culture compared to cells from the periphery [94, 95]. By seeding cells in high density in non-adhering agarose moulds matrices, self-assembled fibrocartilaginous tissues recapitulating the histological and biochemical characteristics of the native meniscus can be engineered [5]. The utilisation of autologous cells is considered safe due to the absence of disease transmission risks and the negligible concern of immunological rejection [96]. However, the clinical translation of this approach presents challenges, as harvested cells necessitate expansion, which consequently leads to longer treatment delays and renders single-step procedures unfeasible [34]. Considering the

aforementioned factors, the utilisation of allogeneic cells may be deemed more favourable. Allogeneic cells have demonstrated to be a safe option, with no significant disparities noted in comparison to autologous cells [97, 98]. Nonetheless, serial subculture of the primary cells *in vitro* downregulates tissue-specific genes and leads to the loss of their native phenotype, in particular for cells from the inner meniscal tissue (rich in collagen type II) where self-repair of the tissue is limited [99]. This problem can be partially addressed using novel cell expansion strategies to better maintain the native phenotype. The inclusion of TGF- β 1 and FGF2 during the expansion of meniscus-derived cells, in combination with hypoxic conditions, has been shown to support a meniscus phenotype [100].

Since MSCs can be expanded *in vitro* while maintaining their fibrochondrogenic potential, they have emerged as a promising alternative cell source, and could potentially be used to acquire large number of cells required for tissue engineering purposes [101-103]. These multipotent cells can be harvested autologously from various locations including bone marrow and infrapatellar fat pad. Although MSC-based therapies for meniscus repair have shown beneficial effects, their tendency to undergo hypertrophic differentiation and trigger mineralisation via endochondral ossification remains a major concern [104]. To overcome this limitation, a co-culture of MSCs and meniscus-derived cells has been proposed, which might help to maintain the desired phenotype while also circumventing the need for large quantities of meniscus-derived cells [105, 106]. Co-culture has been shown to result in a reduction of hypertrophic markers such collagen type X and matrix metalloproteinase-13 (MMP-13) [105]. Although numerous studies have reported the benefit of MSCs and meniscus cells in co-culture, there is still no consensus on the optimal cell ratio or cell culture conditions. Furthermore, their potential to develop a long-term functional meniscus phenotype is yet to be proven. Another approach to reduce hypertrophy has been the culturing of MSCs within meniscus-derived extracellular matrix by taking advantage of its inherent bioactivity [107].

Recently, a subpopulation of stem/progenitor cells were suggested to be present in the meniscus tissue [108-110]. However, the characteristics, marker genes and isolation methods of human meniscus progenitor cells have not yet been fully elucidated. Recent single-cell RNA sequencing work has identified the melanoma cell adhesion molecule ((MCAM)/CD146) as a potential marker shared for endothelial cells and meniscus progenitor cells with multilineage differentiation capacity [111]. Nevertheless, others have argued such named progenitor cells are too similar to pericytes and smooth muscle cells, highlighting the current inability to discriminate based on immunophenotype [112]. More recently, meniscus progenitor cells have been isolated from the vascular and avascular regions using differential fibronectin adherence with trilineage capacity [113, 114]. Such methods have been extensively used for the selection of progenitor cells from the articular cartilage, termed articular cartilage progenitor cells (ACPs) [115, 116]. These ACPs, which are mainly found in the superficial zone of the articular cartilage, can be enriched by differential adhesion to fibronectin thanks to the high levels of expression of integrin alpha5 (CD49e) [117]. Progenitor populations derived from the meniscus have shown significantly higher expression for the integrins CD49b and CD49c compared to non-selected populations [118]. Furthermore, such meniscus progenitor cells derived from the inner and outer region of the meniscus showed superior fibrochondrogenic capacity under hypoxic conditions compared to their mix populations counterparts [114, 118]. While these findings build evidence suggesting that meniscus progenitor cells could represent an attractive cell type for tissue engineering and regenerative medicine, further investigation is necessary to ascertain their regenerative potential and clinical translatability. In a similar vein to primary fibrochondrocytes, exploring the allogeneic utilisation of meniscus progenitor cells holds significant value. Such investigations could substantially enhance their potential for application by increasing availability, reducing costs, and allowing for preselection of cells. Furthermore, studies comparing meniscus progenitor cells to fibrochondrocytes for transplantation are essential.

Bioinks for 3D bioprinting of fibrocartilaginous tissue

A wide variety of different technologies to spatially deposit cell-laden bioinks have emerged in recent years, including extrusion, inkjet, or light-based techniques [119, 120]. In all cases, the properties of the bioink must be carefully tuned not only to ensure compatibility with the bioprinting hardware but also to ensure cell viability and function post-printing [119, 121-123]. This has led to the development of a wide range of different bioinks, from relatively simple cell suspensions to chemically modified polymers containing growth factors [124, 125]. In this section, the different bioinks developed specifically for meniscus tissue engineering will be described.

Due to the high spatial control provided by extrusion-based bioprinting, it is currently the most commonly used 3D bioprinting strategy [126]. The main challenge of extrusion-based approaches is the development of a bioink with rheological properties that match with the biofabrication window, allowing 3D deposition without compromising cell viability [127]. Different rheological parameters including: (1) yield stress, which prevents the spreading of the ink after extrusion, increasing printing fidelity, but too high stress can result in cell damage; (2) shear-thinning, indicating reduced flow resistance with increasing shear rate during extrusion; (3) rapid recovery, which will increase the viscosity of the bioink after extrusion, allowing to obtain high shape fidelity; are commonly investigated to assess the suitability of a specific bioink for 3D bioprinting [128, 129]. Cohen *et al.* were one of the first to demonstrate the ability to encapsulate articular chondrocytes in an alginate-based bioink to create meniscus-shaped structures recreating the shape derived from a CT scan [130]. This approach maintained high cell viability, with a uniform cell distribution, whilst preserving the cells' ability to secrete meniscus extracellular matrix (ECM) components [130, 131]. Alternative deposition methods to extrusion-based bioprinting are also available, including inkjet bioprinting. While inkjet bioprinting have also been applied because of its simplicity and precision, it

presents some limitations when forming a stable structure due to the lack of sufficient viscosity to self-sustain its own weight after plotting [132, 133].

Alginate is a naturally occurring anionic polysaccharide isolated from bacteria and brown algae [134, 135] which is commonly used as a bioink in bioprinting. It is a linear unbranched copolymer of mannuronic acid (M) and guluronic acid (G) linked together in different sequences or blocks. The amount and distribution of the G and M units depend on the source of the alginate, and will define the final physio-chemical properties of the material. Alginate forms a gel through ionic crosslinking between the G blocks of adjacent polymer chains and multivalent cations like Ca^{2+} , creating ionic bridges through interactions with the carboxylic groups [136]. Alginate has been widely used in the field of tissue engineering due to its biocompatibility, non-immunogenicity and its gentle gelation process, enabling cell encapsulation [125, 130, 131, 137, 138]. However, alginate hydrogels present a low *in vivo* degradability, since alginate-degrading enzymes are not present in mammals. Degradation may then occur through an outward flux of ions into the surrounding environment, making this degradation process very unpredictable. In addition, alginate gels exhibit poor cell adhesion and infiltration because they lack of specific molecular ligands to interact with cells. To address these limitations, different modifications have been introduced into the alginate polymer chain. The incorporation of cell adhesion ligands through RGD containing peptides promote cell attachment and interaction [136]. To enhance the biodegradability of the alginate gels, chemical approaches like oxidation or gamma-irradiation of the polymer have been employed [135, 137, 139]. Although such methods increase the degradation rate of alginate, they also have a negative effect on their mechanical properties. Nevertheless, alginate has shown to support fibrochondrogenesis of multipotent cells and fibrochondrocytes both *in vitro* and *in vivo*.

Alginate and agarose-based hydrogels are commonly used to direct MSCs differentiation towards a chondrocyte-like phenotype to recapitulate the inner zone of the

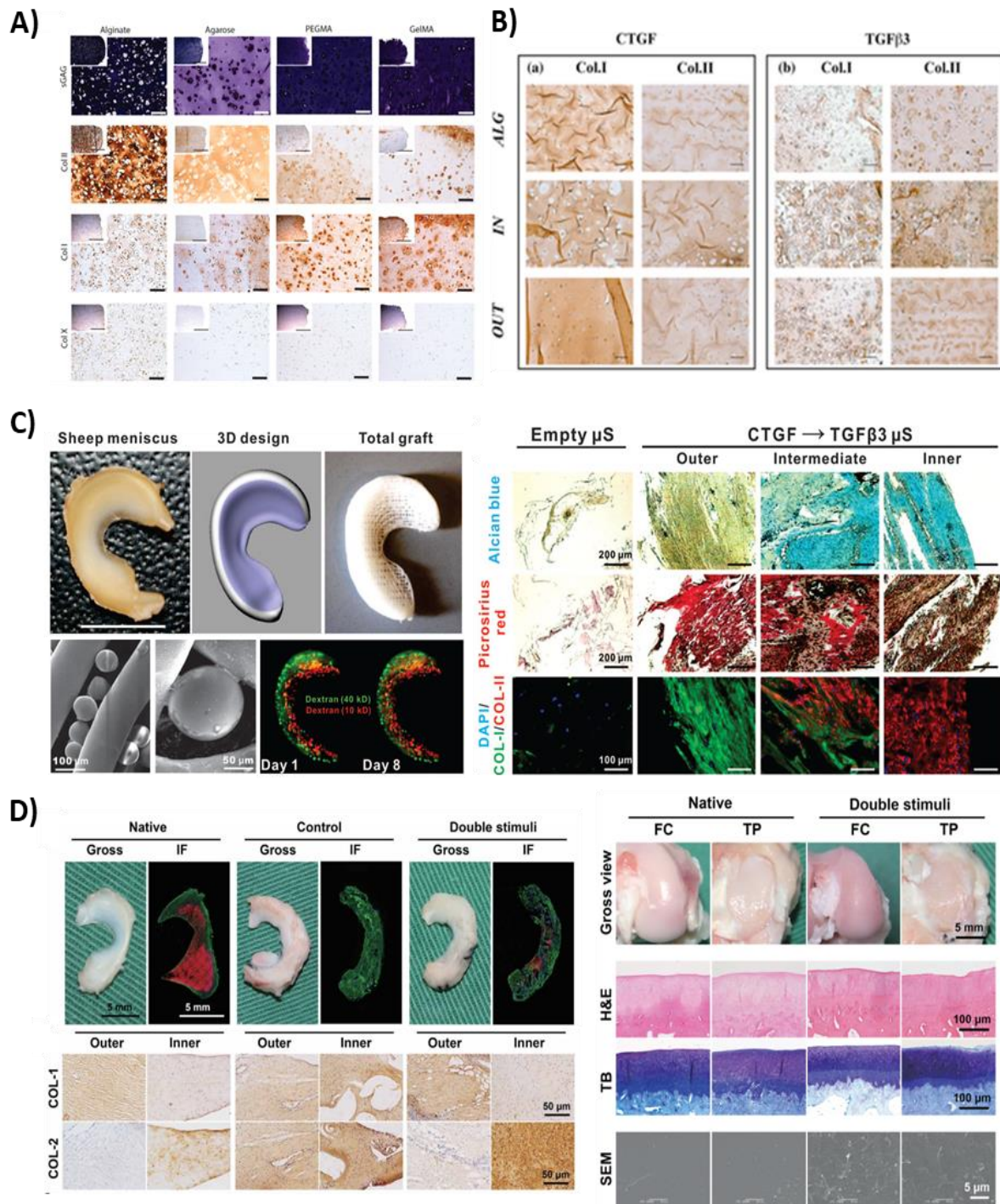


Figure 2.4. Biofabrication of 3D bioprinted implants for meniscus regeneration. (A) Histological analysis of cell-laden hydrogels following *in vitro* culture. Images 10X with 4X inset, scale bar is 100 μ m and 1 mm, respectively [1]. (B) Immunohistochemical analysis on the effect of the meniscus extracellular matrix and exogenous growth factors in cell-laden hydrogels [2]. (C) Left - Fabrication of an anatomically correct meniscus implant (Scale bar is equal to 15 mm) containing PLGA microspheres. Right – Histological evaluation of the implants containing growth factors after 6 weeks of *in vitro* culture [3]. (D) Left - Meniscus regeneration in a rabbit model 24 weeks after implantation (Green - Col I; Red - Col II). Right – Gross and microscopic evaluation of the joint following 24 weeks of implantation. FC: Femoral condyle; TP: Tibial plateau; TB: Toluidine blue [8].

meniscus tissue (Figure 2.4A). The absence of cell-binding motifs within such hydrogels allows the cells to acquire the more rounded cellular morphology associated with the inner zone of the meniscus [1, 140, 141]. In contrast, cells can directly adhere to hydrogels such as gelatine methacryloyl (GelMA), displaying a more fibroblast-like morphology and phenotype [1, 140]. The spatial deposition of these different hydrogels can be used to create distinct domains within 3D printed constructs, which can present regionally different mechanical properties, cell types or factors [142, 143]. All this demonstrates that the biomaterial of choice not only determines the physical properties of tissue engineered scaffold, but also determines the biological properties facilitating proliferation and differentiation into tissue-specific cells.

The ECM extracted from different tissues and organs has also been used to produce tissue-specific bioinks [144, 145]. ECM extracted from the inner and outer zones of the meniscus can be integrated into bioinks to help instruct encapsulated stem/progenitor cells to differentiate into specific meniscus-cell phenotypes [2, 146, 147]. It has been demonstrated that MSC-laden alginate bioinks can be functionalised with inner or outer meniscus ECM and stimulated with factors such as transforming growth factor- β 3 (TGF- β 3) or connective tissue growth factor (CTGF) to promote meniscus region-specific differentiation (Figure 2.4B) [2]. Cells encapsulated within inner ECM-functionalised bioinks displayed a rounded morphology while cells in outer ECM-functionalised hydrogels presented a tendency towards an elongated shape. The presence of the growth factors further supported the chondrogenic or fibroblastic phenotype within inner and outer ECM-functionalised hydrogels. Others have explored the potential of meniscus-ECM functionalised implants *in vivo*, and demonstrated improved biochemical and biomechanical properties when compared to non-functionalised implants, however, the chondroprotective effect of these implants was still incomplete [146]. To improve the structural organisation of bioprinted meniscal grafts, the application of ultrasound to help structurally organise cellular arrays has also been explored [148]. This novel biofabrication approach combined the benefits of 3D bioprinting to entrap cells and

ultrasound to obtain specific cellular organisations within the hydrogels, which allowed them to recreate an alignment similar to native tissue within 3D structures. These different studies demonstrate the benefits of introducing defined patterns of biomaterials and bioactive factors through 3D bioprinting to control meniscal tissue repair.

Although hydrogels mimic the cell microenvironment and support matrix deposition, they are generally mechanically weak compared to native tissues, which often renders them unsuitable for load-bearing applications. The mechanical properties (e.g., stiffness and viscoelasticity) of hydrogels are known to play a key role in determining the phenotype of encapsulated cells [149, 150]. Several strategies have been explored to improve the mechanical properties of hydrogels for tissue engineering applications. To improve biological, mechanical and rheological properties, mechanically different hydrogels have been combined to create interpenetrating networks (IPNs) or reinforced with nanomaterials [151-153]. The combination of crosslinked polymer networks with different mechanical attributes within an IPN (often referred as ionic-covalent entanglement) improves the overall mechanical behaviour of the final hydrogel far beyond their individual components separately [153]. Polylactic acid (PLA) nanofibres and nanofibrillated cellulose have been combined with alginate to 3D bioprint meniscus-shaped structures with high cell viability that also provided an environment supportive of cell proliferation and differentiation [154, 155]. The addition of the PLA nanofibres to an alginate-based bioink was intended to mimic the nanofibrous matrix of native meniscus tissue, however no significant mechanical reinforcement effect was observed using this nanocomposite. The ideal bioink for meniscus tissue engineering should not only allow for the biofabrication step, but also recreate a favourable microenvironment for meniscal regeneration. While different bioinks have been developed that partially recreate the native cellular microenvironment, they still fail to mimic the complex architecture and mechanical behaviour of the meniscal fibrocartilaginous tissue. Future work will benefit from the use of multiple bioinks in order to recreate the distinct zones of the meniscus by

using different biomaterials with specific bioactivities or by precisely incorporating growth factors or other signalling molecules, which are discussed below.

Signalling to spatially control meniscal tissue formation

Different physio-chemical stimuli, ranging from growth factor proteins to cyclic compressive mechanical stress, have been used to trigger specific biological pathways in cells to enhance the functional properties of engineered tissues. As stated previously, a critical advantage of bioprinting as a biofabrication technique is the precise spatial delivery of growth factors, motifs for enhanced cell interaction or ECM components. In the native tissue, the expression of CTGF is triggered under mechanical stress and regulates the expression of collagen type I [156]. The application of exogenous CTGF has been used to encourage a fibrochondrogenic phenotype, elevating the levels of collagen type I in human MSCs [3, 157, 158]. Fibrochondrocytes share characteristics of both chondrocytes and fibroblasts, producing collagen type II and type I. Growth factors of the TGF- β family are generally added to cell culture growth media to induce chondrogenesis, stimulating collagen and proteoglycan production via SMAD2/3 phosphorylation [95, 110, 159-161]. The sequential delivery of CTGF and TGF- β 3 has been shown to promote a fibrocartilage phenotype similar to the native meniscus, with the engineered tissue stained positive for collagen type I and type II [3, 157]. Such growth factors can be incorporated into 3D (bio)printed constructs and released in a controlled manner [162, 163]. By spatially controlling the position of CTGF and TGF- β 3 in 3D printed meniscus-shaped scaffolds, studies have been able to recapitulate the zone-specific matrix phenotypes of the native tissue: an outer zone rich in collagen type I and an inner zone rich in collagen type II [3, 164]. In a meniscus explant model, a high dose of 1,000 ng/mL of CTGF in combination with a slow and sustained release of TGF- β 3 (0.29 ± 0.1 ng/day) induced healing of avascular meniscus tears via stem cell recruitment [165]. Another growth factor commonly used in meniscus tissue engineering is fibroblast growth factor-2 (FGF2), which has been shown to act as a potent stimulator of

proliferation and chondrogenesis [166-168]. Kasemkijwattana *et al.* assessed the effect of different growth factors on fibrochondrocytes, reporting enhanced proliferation and collagen synthesis with epidermal growth factor (EGF), FGF and platelet derived growth factor (PDGF), while no effects were noted using insulin-like growth factor-1 (IGF-1) [167]. The spatiotemporal delivery of factors via 3D bioprinting, therefore, represents an attractive approach to meniscus tissue regeneration, with the time course strongly influencing the success of such approaches [169].

A common challenge in cartilage and fibrocartilage tissue engineering is the development of highly organised, collagen-rich constructs with biomimetic mechanical properties. Proteoglycans contribute to the tissue's high-water content and enables the resistance to large compressive forces generated within the knee. However, their presence is known to impact collagen fibril morphology and formation kinetics by hindering lateral growth during fibril formation [170, 171]. Furthermore, a negative correlation between sGAG content and collagen fibre formation during *in vitro* chondrogenesis has been observed [172]. Such findings have supported the use of catabolic enzymes such as chondroitinase ABC (cABC), which cleave GAGs, as biochemical stimuli capable of inducing the reorganisation of collagen fibres and hence enhancing the mechanical integrity of the tissue. The mechanical properties of engineered neomeniscus tissues can be greatly improved by the dual application of cABC and TGF- β [173-175]. This combination of factors results in a denser collagen matrix, with associated increases in fibre alignment and tensile strength without compromising the compressive strength of the tissue [176]. Lysyl oxidases (LOXs) are enzymes that catalyse the modification of collagen fibres and induce the formation of covalent cross-links between fibres, thus increasing the biomechanical properties of the tissue [177]. Although LOX enzymes can be triggered under hypoxic conditions, their exogenous administration also enhances the functional maturation of collagen-based tissues both *in vitro* and *in vivo*. An increase in collagen density and crosslinking via cABC and LOX, and the matrix synthesis stimulator TGF- β 1, has been shown to enhance the tensile modulus by 186% compared to control

conditions [170]. Such a combination of bioactive factors, therefore, can effectively enhance the mechanical properties of the resulting fibrocartilaginous tissue. To further increase the tensile strength of engineered tissues, Gonzalez-Leon *et al.* investigated the application of lysophosphatidic acid, a bioactive phospholipid that induces cell contraction, to promote directional remodelling that resulted in an improvement in the tensile anisotropy of self-assembled fibrocartilaginous tissue [178]. All these are examples how it is possible to modulate the ECM of engineered tissues to improve collagen fibre formation and the resulting mechanical properties.

As stated previously, *in situ* meniscus tissue resists the mechanical stresses of the joint environment and is sensitive to mechanical forces [91]. Different compression and tensile regimes have been investigated to identify the specific mechanotransductive pathways regulating neo-fibrocartilage formation and maturation, which have been extensively reviewed elsewhere [91, 179, 180]. Previous research on the mechano-responsiveness of fibro- and articular chondrocytes revealed that dynamic stimulation not only regulates the expression of ECM-related genes but also modulates the inflammatory and catabolic signals [90, 181]. For example, when a loading regime of 10% axial strain at 1 Hz frequency for 1 hour per day, with alternating cycles of 1 minute of dynamic stimulation and resting periods are applied, studies found a significant increase in the biomechanical and biochemical properties of self-assembled meniscus constructs [182].

Mechanical stimulation can also induce the alignment of the collagen fibres, enhancing the tensile or compressive properties of the engineered tissues. Therefore, it is reasonable to assume that bioreactor-based maturation protocols, where bioprinted constructs are allowed to develop post-printing, will form the basis of many successful meniscus tissue engineering strategies. In one of the most advanced studies on 3D bioprinted meniscus implants to date, a PCL scaffold was printed with circumferential and radial fibres to mimic the microstructure of native meniscus tissue (Figure 2.4D) [8]. Separate application of biomechanical dynamic loading to 10% strain at 1 Hz for 1 hour

per day for 5 days a week, or biochemical stimuli in the form of TGF- β 3 and CTGF over 2 and 4 weeks *in vitro*, failed to induce substantial differences in gene expression between MSCs seeded in the inner and outer zones of the construct. However, the combination of biomechanical and biochemical stimuli produced a synergistic effect, inducing a zonal-specific matrix phenotype and an anisotropic organisation within the engineered fibrocartilage tissue. When implanted in a rabbit model, tissues engineered in the presence of biomechanical and biochemical cues *in vitro* restored the functional anisotropy of the meniscus *in vivo* and stabilised the knee joint. After 24 weeks *in vivo*, only minor surface irregularities were observed within the joint [8, 183]. The results from this study validate not only the use of 3D printing, but also a combination strategy consisting of both biochemical and biophysical cues for the regeneration of this complex tissue. To conclude, future bioprinting strategies should consider introducing a combination of biochemical and biomechanical stimulation regimes post-printing in order to achieve robust neotissues for clinical translation.

2.4.2. 3D (Bio)Printing of biomimetic fibrous scaffolds and fibre-reinforced composites for meniscus tissue engineering

Because the meniscus is a load-bearing tissue, most of the commonly used cell-friendly hydrogels like alginate or GelMA are not mechanically suitable for implantation. The polymer concentrations and densities of hydrogels can be increased to improve their mechanical properties, but this can significantly decrease cell viability and (fibro)chondrogenic differentiation [184]. Hydrogels have been reinforced in the past by depositing a thermoplastic material alongside a cell-laden bioink with low polymer density in order to increase the overall strength of the construct without lowering cell viability and biosynthetic activity [1, 164, 185-187]. This process is often referred to as multiple-tool biofabrication when performed within a single printing process and requires 3D printing devices capable of printing hydrogels *via* inkjet or an extrusion-based system as well as thermoplastics *via* fused deposition modelling (FDM). While current

bioprinting strategies have been partially successful in promoting fibrocartilage tissue formation *in vitro*, their combination with distinct 3D printing processes such as FDM allows for further increases in the complexity of the final construct.

Wide ranges of synthetic polymers are currently under investigation for tissue engineering applications, extending from scaffold fabrication to drug delivery systems. Synthetic polymeric materials offer a number of advantages over other biomaterials including the ability to tailor mechanical properties and degradation kinetics to suit specific requirements. Furthermore, synthetic polymers can be easily modified to incorporate functional groups or to create copolymers with new functionalities. Among the synthetic polyesters, PCL is a widely studied polymer that has been used in a number of Food and Drug Administration (FDA) approved biomedical devices. PCL is hydrophobic and semicrystalline polymer with a melting temperature of 59 to 64°C depending on the molecular weight of the polymer [188]. The low melting temperature of PCL allows the polymer to be easily extruded through fine nozzles upon heat melting and under compressive pressures. Extruded PCL filaments can be arranged to fabricate scaffolds with a wide range of architectures, and to be co-printed with bioinks to build mechanically reinforced cellular constructs [1, 2]. PCL degradation occurs through hydrolytic excision of the polymer at the surface. Although this degradation can be predicted, depending on the molecular weight, total degradation may take up to 4 years *in vivo* [188]. PCL undergoes plastic deformation and failure when exposed to extensive stress that drives the attention for the synthesis of copolymers [189].

In an exemplary study, Lee *et al* described the development of a 3D printed meniscus-shaped scaffolds with spatiotemporal delivery of TGF- β 3 and CTGF capable of inducing endogenous cells to form zone-specific matrix phenotypes (Figure 2.4C) [3]. Here, biomechanical functionality was achieved by altering the architecture of the PCL network that provided structural support to the implant. An ovine *in vivo* study demonstrated successful meniscal tissue formation within the growth factor-containing

scaffolds, but the constructs failed to exert chondroprotective effects on the underlying cartilage [190]. Suggested reasons for this included an inadequate fixation of the implant horns to the tibial plateau, an insufficiently flexible PCL frame, and a lack of control over the postoperative weight-bearing behaviour in the animals. In such scenarios, it may be that damage to the underlying articular cartilage could be prevented by decreasing the compressive stiffness of the printed implant to more closely match that of the native meniscus. Several recent studies have therefore employed PCL fibre architectures in an offset pattern, which significantly decreases the compressive modulus of the resulting scaffolds [42, 183, 191]. Furthermore, to better mimic the bi-phasic nature of soft tissues such as the meniscus, several studies have sought to combine a PCL scaffold with a hydrogel or bioink to provide a more viscoelastic mechanical response. For example, the combination of appropriately designed 3D printed PCL networks with interpenetrating network hydrogels has been shown to result in synergistic increases in construct stiffness and the development of more biomimetic mechanical properties [191]. A further advantage of 3D printing for meniscal tissue engineering is that the spatial complexity of the tissue can be considered, for example by the inclusion of circumferentially oriented fibres, which has been shown to support the development of constructs with more biomimetic compressive and tensile mechanical properties [142]. Furthermore, to obtain zone-specific meniscus phenotypes, such constructs can be seeded with region specific hydrogels/bioinks to support the development of an extracellular matrix that is more characteristic of either the inner or outer zone of the meniscus. All these different studies demonstrate that composite biomaterials can be used to recreate some of the biological and biomechanical properties of the meniscus tissue. However, most of the currently reported 3D printed implants possess a low ratio of the tensile to compressive modulus (i.e., they are generally not stiff enough in tension and are overly stiff in compression), a property that is integral to the biomechanics of the tissue and required for the correct functionality of the native meniscus. Future studies should explore the use of different architectures to produce implants with anisotropic mechanical properties more mimetic

of the meniscus, which will contribute to implant integration and the restoration of normal joint biomechanics. Furthermore, it has been highlighted that scaffold microarchitecture not only plays a role in determining implant mechanical properties but also regulates collagen organisation within the engineered tissues [192]. Such results emphasise the need to investigate 3D printed meniscus implants, which not only provide specific mechanical properties, but also function to guide the growth and anisotropy of the engineered tissue.

2.4.3. Melt electrowriting (MEW)

The more recently developed melt electrowriting (MEW) technique, which merges principles from electrospinning and additive manufacturing, further increases the resolution of the deposited thermopolymers fibres, from 100-150 μm that is possible with FDM, to fibre diameters in the submicrometric range (Figure 2.5) [193, 194]. The small diameter of MEW fibres can regulate cell morphology and phenotype, while increased scaffold surface area facilitates cellular infiltration and neo-tissue formation [6, 195]. Its use for tissue engineering applications is of great interest since it enables the

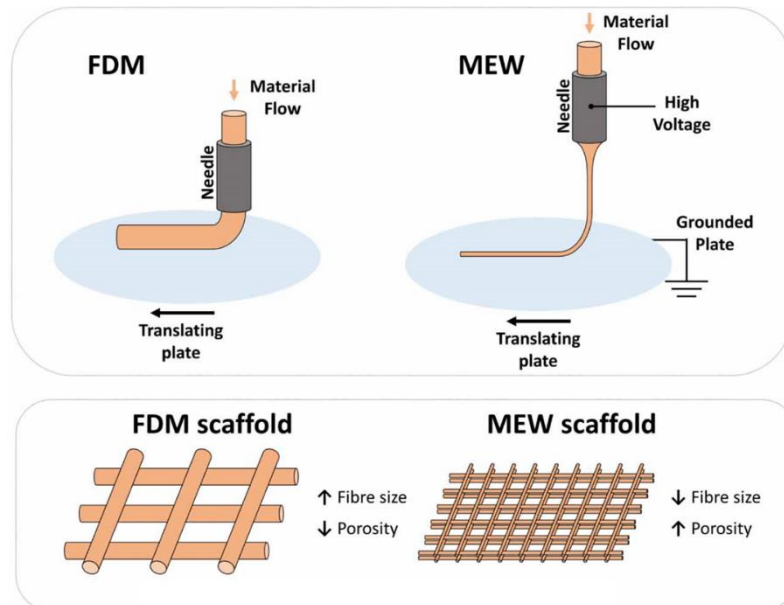


Figure 2.5. Schematic diagram of fused deposition modelling (FDM) and melt electrowriting (MEW) [6].

biofabrication of scaffolds with complex microarchitectures able to mimic the organisation and biomechanics of cartilaginous tissues when reinforced with a hydrogel [186, 196, 197].

In this technology, a melted polymer is extruded through a metal needle, often termed nozzle, with direct writing of fibres being facilitated by the controlled movement of the collector and/or print head. A potential difference between the nozzle and the collector plate leads to the formation of an electric field which stabilises the fluid flow, forming a continuous fibre. The electrified jet can then travel linearly toward a grounded or oppositely charged collector, and be sustained at very low flow rates which allows low fibre diameters. Using highly viscous melts instead of polymer solutions, as traditionally used in electrospinning, significantly improves the control and stability of the jet [198]. The movement of the collector and print head follows pre-programmed movements, which allows high-resolution polymer deposition into well-defined structures.

Depending on the printing parameters, the resulting fibre diameter can be controlled, adjusted, and deposited following different configurations. By tuning the geometry of the deposited fibre pattern, the resulting structures can be tailored to display complex mechanical behaviours replicating the anisotropic nature of human tissues [199, 200]. The scaffold architecture can then be used not only to tune the mechanical properties to approximate desired functionalities but to regulate cellular arrangement and behaviour [195, 201, 202]. Cellular distribution in squared geometries results in randomly arranged cells without any preferential direction. On the other hand, scaffolds with a rectangular architecture can promote cellular alignment following the preferential direction of the scaffold fibres [203]. The great degree of dynamic control over fibre diameter allows the fabrication of complex structures with a varied spectrum of fibre diameters by altering parameters such as pressure and collector speed [204]. Such an approach can be used to obtain heterogeneous scaffolds which can regulate cell growth and elongation by controlling fibre diameter in the longitudinal and transversal directions [205].

One major drawback associated with the use of MEW for tissue engineering applications has been the limited print height which can be achieved; this is due to the residual charges within printed fibres that remain with increasing build height which reduces the accuracy of the fibre placement. Such distortion is explained by a decline in the electrostatic forces, which results in enhanced material accumulation of polymer at the tip of the nozzle reducing the control and stability of the jet. Such inhomogeneous deposition initiates further defects in the following build layers resulting in a distorted scaffold. To overcome this thickness limitation, recent studies have shown that iterative adjustment on the z-axis and increasing the applied voltage during the MEW process, is key to maintain a constant electric force during the printing process which can yield scaffold heights exceeding 7 mm [206]. This demonstration has increased the attractiveness of the technology for biomedical applications as large scaffolds with porosities over 97% can be obtained which resulted in beneficial *in vivo* bone regeneration when compared to traditional additive manufacturing approaches [6, 207].

It is known that the “ideal” tissue-specific scaffold should support appropriate biological responses via cell-scaffold interactions, whilst providing mechanical and structural support. In MEW, cell-scaffold interactions are strongly linked and influenced by fibre diameter, spacing, and geometry. Notably, countless geometries are accessible by taking advantage of the high degree of freedom and customisation that this technology offers including microfabrication on non-planar surfaces or rotating mandrels elevating the complexity of the MEW structures that can be obtained [208-210]. A recent study has shown the possibility to design and print scaffolds that replicate the round rim and wedged-shaped geometry of the meniscus, and the successful incorporation of the cell component *in vitro* [41]. This is the first time MEW is applied for the regeneration of the semilunar fibrocartilaginous tissue. Taken together, this indicates that MEW will be particularly useful in the biofabrication of biomimetic fibrous scaffolds for the regeneration of musculoskeletal tissues such as the meniscus.

2.4.4. Emerging strategies

Until now, conventional layer-by-layer biofabrication strategies require long processing times that could hamper the translation of bioprinting in the clinic for relatively large anatomical constructs like cellular menisci. Recently, an optical-tomography-inspired approach has been applied to generate cell-laden constructs that maintain good cell viability [211, 212]. The final 3D printed object is obtained through the superposition of multiple 2D images projected from different angles. By using photocrosslinkable polymers, this new strategy can create large and complex 3D structures faster than conventional additive manufacturing techniques without the need for supports, which could benefit the translation of bioprinting into the clinic. This technology has been brought further by demonstrating the ability to incorporate multiple materials and cells, while its convergence with MEW enhanced the mechanical behaviour of the resultant constructs [213]. These findings provide exciting opportunities for advanced biofabrication strategies which will have as a main challenge to preserve the processing speed while increasing the micro- and submicroscale resolution [214].

2.5. Biomimicking developmental processes for meniscus tissue engineering

Although bioprinting has been successfully employed to recreate some of the features seen in native meniscus, complete recapitulation of many architectural features key for the correct functionality of the meniscus remains elusive. This has been suggested to be caused, at least in part, by the presence of supporting hydrogels used in many bioprinting strategies, which can limit the capacity of cells to interact, remodel and organise within engineered tissues [215, 216]. In light of this, scaffold-free approaches such as cellular self-assembly and self-organisation have emerged as a means of generating highly biomimetic engineered tissues.

Self-assembly procedures aim to replicate the developmental processes occurring naturally in all living organisms. The concept relies on the cell's ability to spontaneously organise driven by cell-cell interactions without the intervention of external forces (e.g., cells cultured on non-adherent substrates are induced to interact to each other to minimise the system's free energy) [217]. It has been proposed that the self-assembly process is mediated by cadherin-cadherin interactions [218]. Similarly, self-organisation approaches aim to exploit cell's ability to synthesise neo-tissue by maximising cell-cell interaction. The organisation, however, appears by external inputs into the system (e.g., bioprinting will create an organised system, and then cells are expected to secrete ECM forming a continuous organised tissue) [216, 217]. Pellet culture is an effective method to force cell aggregation by an initial centrifugation step, thus it is considered a self-organised approach where cell aggregates are induced by the initial rotational force. This scaled-down approach increases the degree of cell-cell interaction and facilitates the formation of meniscus-like microtissues [105, 106].

Clinically relevant self-assembled meniscus constructs have been explored by co-culturing articular chondrocytes and meniscus fibrochondrocytes into agarose moulds [5, 219]. The co-culture system yields a matrix composed of collagen type I and type II. Furthermore, the self-assembly within circular-shaped agarose moulds induced a preferential direction for the collagen alignment. As a result, after 8 weeks of culture, the self-assembled tissue presented a significantly higher tensile modulus (226 ± 76 kPa) than when tested in the radial direction (67 ± 32 kPa), resembling the meniscus anisotropy [5]. The application of TGF- β 1, cABC, LOX and different mechanical stimulation regimes, previously discussed, have been shown to improve the functionality of these self-assembled tissues, approaching the biochemical composition and biomechanical properties of native meniscus [181].

Translating or developing 3D bioprinting approaches where cells are able to self-organise within predefined patterns and geometries would be a powerful technology to

recapitulate the organisation and microarchitectures of native tissues [220]. Recently, Daly *et al.* reported the use of inkjet bioprinting to deposit a defined number of cells into 3D printed PCL microchambers. These inkjetted cells rapidly condensed into cell spheroids due to the hydrophobicity of the PCL microfibres [221]. The polymeric microchambers provided boundary conditions to the cell growth that eventually developed into a stratified cartilage tissue with a depth-dependent collagen fibre architecture, mimicking the structure of native articular cartilage. Such a study is an example on how cell self-organisation ability can be guided through geometrically defined physical boundaries, thus realising specific engineered 3D environments as a way to control the growth and arrangement of cellular structures.

2.6. Bioprinting of structurally organised tissues: Setting boundaries

The functionality of the meniscus is closely related to its intricate and highly specialised ECM architecture [40, 222]. Most collagen fibres are oriented circumferentially, bearing the joint loads that are converted into hoop stresses within the tissue, thus protecting the underlying articular cartilage. Tissue engineering typically aims to employ scaffolds to provide initial structural support for cell adhesion to serve as a template for tissue formation aiming to restore, maintain or improve damaged tissues. Therefore, much focus has been given to replicating the physiological functionality of the meniscus by using a scaffold with *in vivo*-like anisotropic behaviours [42]. Although partial success in replicating the complex mechanical behaviour has been achieved, limitations associated with such biomaterials, such as high stiffness, rapid plastic deformation and an inability to replicate the anisotropic and non-linear properties of the native tissue, motivate the development of novel strategies. The engineering of tissues with biomimetic collagen architecture and ECM distribution would represent a powerful approach to overcome such limitations. However, the control over tissue organisation *in vitro* remains a challenge.

A growing number of bioprinting strategies have investigated recreating the shape of the tissue target (e.g., the semilunar shape of the meniscus) [132, 143]. However, the reproduction of the anatomical shape does not ensure functionality, which is often defined by the hierarchical spatial arrangement of cells and ECM components within the tissue [223]. Many studies have shown how living cells can sense, respond and migrate following mechanical, biochemical, and geometrical cues. Among these different factors, spatial or physical confinement has raised special interest in light of the ability to influence individual and collective cell behaviours with great control [224, 225].

Cells within tissues and organs are embedded into highly organised microenvironments (e.g., collagen fibres, neighbouring cells...) imposing specific boundary conditions which influence the cell architecture and functionality. Biophysical parameters such as cell shape can switch cell fate between different biological processes like growth, differentiation, or apoptosis [226]. In this context, micropatterning strategies allow for the control of architectural and boundary conditions. For example, MSCs treated with TGF- β 3 differentiate into chondrocytes when plated on small micropatterns, but into smooth muscle cells when plated on larger micropatterns, indicating that cell shape can regulate cell fate [227]. Furthermore, the increased spreading of chondrocytes during *in vitro* culture is directly correlated with enhanced dedifferentiation [228]. By changing the anisotropy of the shape pattern, it is possible to control the shape, alignment, and directionality of single cells [229-231]. All this indicates that topography on flat surfaces can play an important role as boundaries by limiting cell spreading and mimicking physiological confinement [232]. Along these boundaries, the cytoskeleton filaments can be anchored, aligned and reoriented [225].

Topographical features have an important role in affecting cell alignment (e.g., contact guidance on anisotropic surfaces). Such an effect has been previously explored by using elongated grooves through which cells align parallel to the long axis [233, 234]. Both biological (via focal adhesions and actin microfilaments) and physical factors have

been proposed as mechanisms driving the contact guidance of cells [235, 236]. Similarly, electrospun fibres can instruct and guide cell growth and phenotype, and direct subsequent tissue formation providing contact guidance to cells [237, 238]. These scaffolds show high mechanical strengths compared to hydrogels or tissues engineering via self-assembly of cells, and can provide topographical cues to align cells which in turn directs matrix deposition in a similar direction [239]. Building from this, multi-layered electrospun scaffolds presenting circumferential or radial alignment seeded with MSCs were able to recapitulate multi-layered collagen fibre alignment dictated by the scaffold fibre alignment [240]. However, the dense 3D arrangement hinders cell invasion and minimises diffusion, which can be at least partially addressed by the introduction of sacrificial materials to increase construct porosity [241]. Although being a highly effective strategy to promote cell alignment, this technology is still very limited for the development of a meniscus substitute scaffold due to the limited control of pore size and construct thickness.

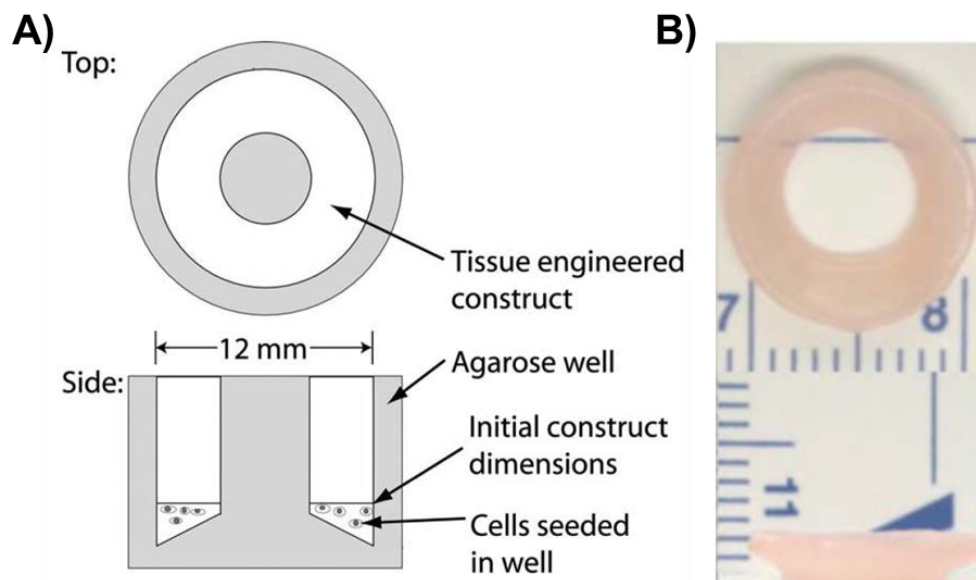


Figure 2.6. Scaffold-free method for growing meniscus-shaped constructs [5]. (A) Schematic diagram of the agarose wells. (B) Gross morphology of the cellular construct at 4 weeks. Scale marking is in mm.

Given the sensitivity of cellular self-organisation processes to spatial boundary conditions, their appropriate implementation into tissue engineering strategies represents

a promising approach for controlling and directing neotissue organisation. In this context, cell aggregates, although effective to differentiate cells towards a chondrocyte phenotype, present uncontrolled and nonhomogeneous shapes when cultured without the use of an external support structure to retain desired sizes and shapes due to cell-mediated contractions [219]. The extent of such contraction is coupled to the selected cell type and culture conditions [242, 243]. This can potentially be addressed by recognising that cells can sense and respond to geometrical and physical cues. For example, cells localised into non-adherent moulds with a centre post are believed to generate forces that result in the circumferential alignment of the collagen fibres (Figure 2.6) [5]. Such engineered fibrocartilaginous tissues also had a significantly higher tensile modulus in the circumferential (226 ± 76 kPa) than in the radial direction (67 ± 32 kPa), thus recapitulating the anisotropic behaviour of the meniscus tissue. Spatial organisation recapitulating the features of the collagen zonal organisation of the articular cartilage has been achieved by means of microtissues fusion, which are defined as a body of cells that have been driven towards a specific phenotype *in vitro* [7, 215]. Such an approach has shown superior shape fidelity to engineer cartilage grafts when compared to single-cell approaches [173]. The inclusion of a 3D printed framework, which imposed boundary conditions on the developing engineered tissue, was found to guide the tissue growth resulting in a collagen network that highly mimicked that seen in the native articular cartilage (Figure 2.7) [7]. This work demonstrates that by introducing defined and optimised boundaries, the cells' self-organisation can be controlled and directed to replicate native tissue architectures.

Recently, 3D printed lattices have been integrated with inkjet bioprinting of a cell suspension to form spheroids *in situ* upon spontaneous cell aggregation [221, 244, 245]. The polymeric microwells acted as physical boundaries on the growing spheroids directing their growth upwards and supporting the formation of stratified cartilage with biomimetic collagen spatial organisation and mechanical properties. Such a combination of 3D bioprinting technologies demonstrates that such can be used not only to impose defined physical boundaries to direct cell growth but also to precisely locate cells in desired locations.

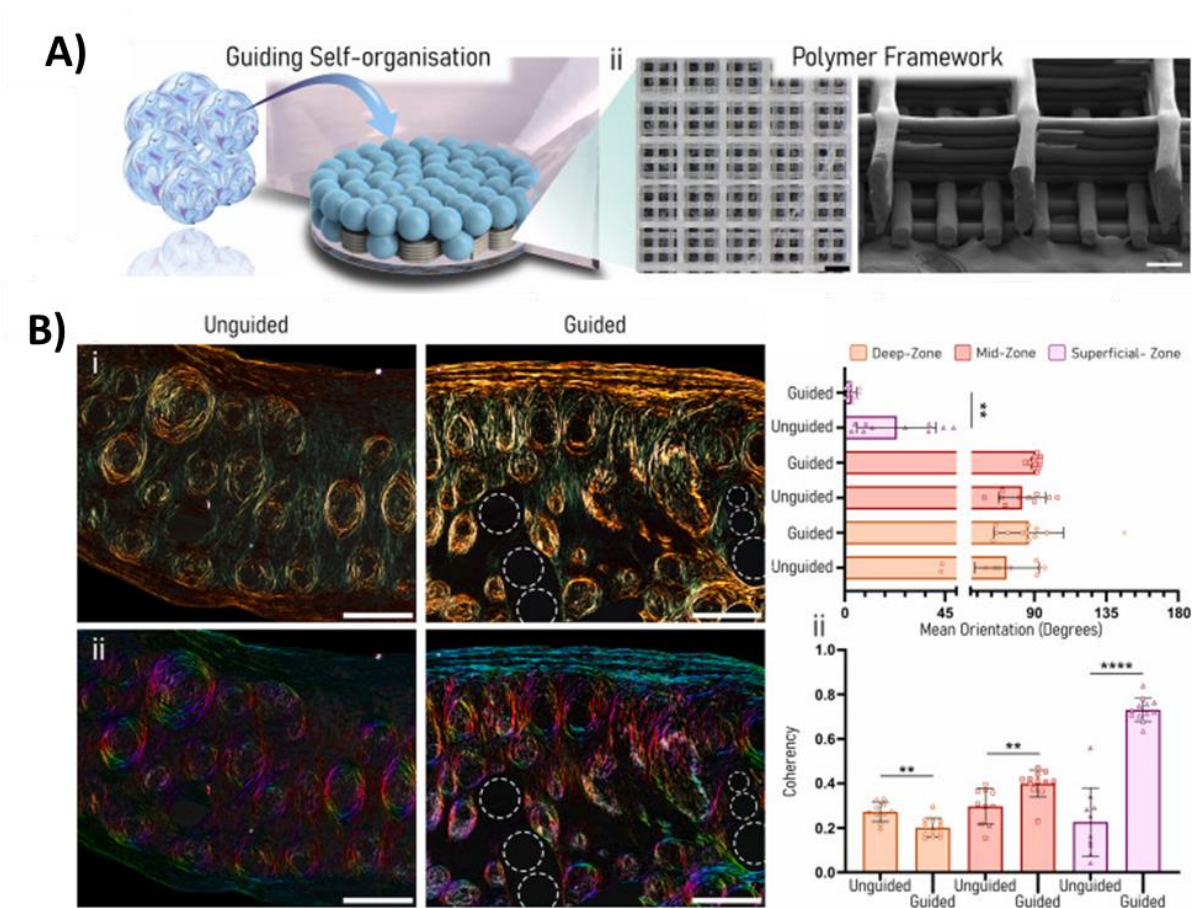


Figure 2.7. The zonal organisation of fusing microtissues can be guided using a 3D printed framework [7]. (A) Schematic, microscopic (scale bar is 500 μm) and SEM (scale bar is 200 μm) images of the 3D printed polymer framework. (B) Polarised-light microscopy (PLM) showing the comparison of the spatial organisation of the collagen network formed under unguided (free self-organisation) and guided (with the addition of a 3D printed polymeric framework) conditions (scale bar is 250 μm).

The extrusion bioprinting of high-density stem cells and organoids within a hydrogel matrix was used to control the self-organisation process which followed the geometry and constraints imposed by the printing process [246]. Those printed structures, after fusion of their cellular building blocks, were able to recapitulate native-like features such as lumens, branched vasculature, and crypts from a millimetre to a centimetre scale. Another 3D printing strategy to introduce architectural cues within engineered tissues is the use of sacrificial inks embedded within a bulk hydrogel [247]. The embedding of these sacrificial templates within bulk hydrogels provided control over the spatial and temporal introduction of such architectural features by controlling the dissolution profile of the sacrificial ink. These findings suggest that boundary conditions can be precisely spatiotemporally controlled to program the architectural organisation of cells and tissues *in vitro*.

2.7. Summary

One of the major challenges with current meniscus tissue engineering strategies is that the implants are generally homogenous and devoid of the zonal complexity of the native tissue, which is integral to the biomechanics of the tissue and hence the overall function and stability of the knee joint. 3D bioprinting holds great potential for engineering biomimetic, highly organised meniscus constructs, which can be custom-made based on medical imaging data. By using medical imaging data, such as MRI or CT scans, it is possible to create implants that precisely match the patient's specific meniscus dimensions, shape, and orientation. By precisely matching the geometry and contours of the patient's meniscus, the implant can fit seamlessly into the joint, reducing the risk of implant migration or instability [248]. Despite the great advancements in bioprinting to produce fibrocartilaginous constructs, engineering human-scale tissues mimicking the structure, composition, and function of the native tissue remains a challenge. The identification of optimal culture conditions, including the use of dynamic bioreactors and growth factors will likely play a critical role in the successful development of such

bioprinted implants, as elegantly demonstrated by the recent study of Zhang et al. [8]. Integrating learnings from other disciplines into the design and development of bioprinted implants, including those from joint and meniscus development, stem cell mechanobiology, and regenerative immunology, will likely play a key role in the success or otherwise of these tissue engineering strategies [55, 91, 249]. Additionally, the application of smart materials, which provide specific functionality or cues upon receiving an external or internal stimulus, such as drug release controlled by the application of physiological load, could be integrated into future 3D bioprinting strategies to engineer dynamic meniscal implants [250, 251]. Finally, it should be noted that the many logistical, regulatory, and commercial challenges that have impacted the clinical translation of tissue engineered products in the field of orthopaedic medicine will also apply to 3D bioprinted constructs. Indeed, the potential for ‘patient-specific’ 3D bioprinted implants will raise new regulatory questions that will urgently need to be addressed if a clinical translation is to proceed in the near future. The development of bioprinted meniscus constructs involves navigating through complex regulatory pathways to ensure safety, efficacy, and compliance with quality standards [252]. Additionally, cost-effectiveness and scalability of the manufacturing process, as well as considerations related to clinical implementation and market acceptance, are important factors that need to be addressed for successful translation into the industry [253].

Chapter 3: Tuning the degradation rate of alginate-based bioinks for bioprinting functional cartilage tissue

3.1. Introduction

Tissue engineering aims to regenerate damaged or diseased tissues by combining supportive biomaterials, biochemical and/or physical signals, and cells, often referred to as the tissue engineering triad [254, 255]. Many tissue engineering strategies rely on the use of a biocompatible polymeric scaffold in which cells deposit the extracellular matrix (ECM) of interest. These polymeric matrices are expected to provide a conducive environment for the cells to proliferate and differentiate [127, 256]. 3D bioprinting is an emerging biofabrication technique enabling the engineering of constructs mimicking the shape, spatial composition, and organisation of complex tissues [1, 126, 257, 258]. Extrusion-based 3D bioprinting strategies have undergone extensive development in recent years and are now widely used for producing 3D tissue constructs [126]. To enable this, numerous different hydrogel inks have been developed, including synthetic (e.g., poly(ethylene glycol) and pluronic) and natural polymers (e.g., alginate and agarose), protein-based (e.g., collagen and fibrinogen) and native tissue-derived materials (e.g., tissue-specific extracellular matrix) [43, 125, 142, 143, 259]. A common goal when designing biomaterials for tissue engineering applications is that they are gradually replaced with newly deposited ECM, without detrimentally affecting the structural stability of the overall construct [260, 261]. In the field of cartilage tissue engineering, it is known that *in vitro* and *in vivo* tissue development is strongly dependent on the rate at which the supporting hydrogel degrades [262-264]. However, little is known about how the degradation kinetics of bioinks will impact the development of bioprinted

cartilaginous tissues, or how material modifications introduced to alter the rate of degradation might impact bioink rheology and hence their printability.

Alginate is a natural polymer commonly used in the 3D bioprinting of engineered cartilaginous tissues [1, 137, 162, 265]. It undergoes a relatively rapid and gentle gelation through the interaction with divalent cations, which makes it attractive for cell encapsulation [266]. A potential limitation of alginate for certain bioprinting and tissue engineering applications is that mammalian cells do not produce enzymes capable of degrading the polymer, leading to a slow degradation process that is primarily controlled by the outward flux of ions into the surrounding environment. In orthopaedic applications, it has been shown that large amounts of residual alginate are still present 8 weeks after *in vivo* implantation, demonstrating the slow rate at which this material degrades [267]. A number of strategies have been investigated to develop alginate-based hydrogels with more controlled degradation kinetics, such as the use of gamma irradiation, oxidation, lower polymer densities, or the use of calcium chelating compounds such as sodium citrate to induce the dissolution of the hydrogel [135, 268-270]. Although such approaches can be leveraged to accelerate the degradation of the hydrogel, balancing the rate of biomaterial degradation with the rate of newly deposited ECM is challenging and tissue specific. In the context of developing an alginate-based biomaterial for 3D bioprinting of cartilaginous tissues, an ink should not only be cell-compatible and printable but should also degrade at an appropriate rate. The degradation should be fast enough to provide space for cells to deposit abundant new ECM, however, it should not be excessively fast such that the printed construct will lose its mechanical integrity and initial structure (Figure 3.1A).

In the present study, we investigated the development of alginate-based bioinks with different degradation rates by combining networks of partially oxidised and raw alginates capable of retaining the 3D printed structure while simultaneously providing an environment supportive of rapid cartilage tissue development. We also explored how the

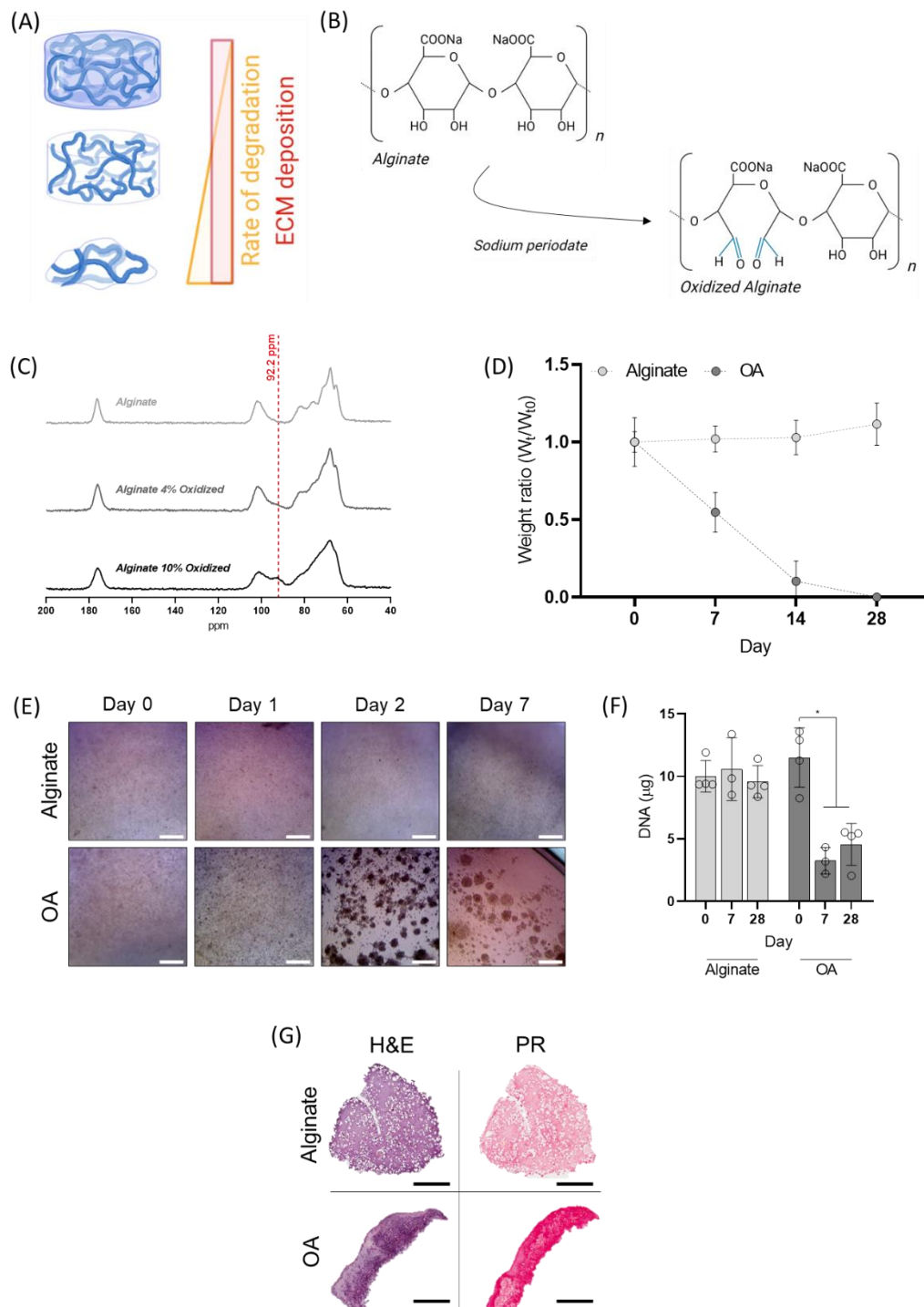


Figure 3.1. Oxidised alginate hydrogels. (A) ECM deposition in hydrogels presenting different degradation rates. (B) Reaction scheme of the alginate oxidation using sodium periodate. (C) ^{13}C NMR spectra for alginates with different degradation rates. (D) Dry weight of oxidised alginate and raw alginate at various time points. (E) Macroscopic views of the cell-laden hydrogels at different time points. The scale bars are equal to 500 μm . (F) DNA quantification in each construct at Day 0, Day 7, and Day 28. (G) Haematoxylin and eosin (H&E) and picosirius red (PR) staining after 28 days in culture. The scale bars are equal to 1 mm. All error bars denote standard deviation, significance was considered $*p \leq 0.05$, $n = 4$.

inclusion of temporary viscosity modifiers (specifically gelatine) would influence the printability of such alginate-based inks. After characterising the degradability, mechanical properties, and biocompatibility of various bioink formulations, we then assessed their relative capacity to support chondrogenesis of mesenchymal stem/stromal cells (MSCs) and the development of functional cartilage tissues *in vitro*.

3.2. Material and methods

Oxidised Alginate (OA) Synthesis: OA was prepared as previously described [271]. Briefly, 1 g of MVG sodium alginate (Pronova Biopolymers) was dissolved in 90 mL of ultrapure deionised water overnight, and mixed with 10 mL of sodium periodate (Honeywell) at different concentrations to achieve different degrees of theoretical alginate oxidation (Table 3.1). The solution was continuously stirred in the dark at room temperature (RT) for 24 h, followed by a dialysis step against deionised water for 3 days (MWCO 3500 Dalton; Fischer). The solution was then sterile filtered through a 0.22 µm filter, after which it was lyophilised. The increased degree of oxidation was assessed through NMR spectroscopy (Bruker Avance II) at RT.

Table 3.1. Theoretical calculations for alginate oxidation.

Name	Theoretical Oxidation (%)*	Amount of NaIO ₄ (g)
Alginate	0	0
OA 1%	1	0.0108
OA 4%	4	0.0432
OA 5%	5	0.054
OA 10%	10	0.108

* Calculations were based on that the molecular weight of the alginate repeating unit is 198 g/mol [271].

Isolation and Expansion of Mesenchymal Stem/Stromal Cells: Bone marrow-derived MSCs were obtained from the femur of a 4-month old pig as previously reported [160]. Briefly, under sterile conditions, the bone marrow was removed from the femoral shaft, and mixed with expansion medium (XPAN), consisting of hgDMEM supplemented with 10% v/v FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL

amphotericin B (all Gibco, Biosciences). A homogenous suspension was achieved by triturating with a 16G needle. The suspension was then filtered through a 40 µm nylon mesh. Cells were counted and seeded into dishes for colony forming assay, or into T175 flasks for expansion. All expansion was conducted in hypoxic conditions at 5% pO₂, and using XPAN medium containing 5 ng/mL FGF2 (Peprotech) [272]. The tripotentiality of isolated MSCs was assessed using chondrogenic, osteogenic and adipogenic differentiation assays. Cells of passage 3 were used for experiments.

3D Bioprinting Process: Cell-laden bioinks were prepared by mixing a 5.3% (w/v) alginate solution and a 21.5% (w/v) gelatine solution (Gelatine type B; Sigma-Aldrich) with a cell suspension (everything in hgDMEM) to obtain a final solution of 3.5% (w/v) of alginate and 5% (w/v) of gelatine containing 20×10⁶ cells/mL. For acellular characterisation, the volume of cell suspension was substituted for hgDMEM to maintain the same polymer concentrations. The different bioinks were loaded into a syringe, and then printed using the 3D Discovery bioprinter (RegenHU) at 16 °C, using a pressure of 0.125 MPa, a translational speed of 4 mm/s and a plastic conical needle of 25G (Adhesive Dispensing). Cellular constructs were printed following a cylindrical geometry of 4 mm in diameter by 4 mm height, following a grid pattern with strand distance of 0.250 mm and a z-distance increase of 0.250 mm. After printing, the different samples were incubated for 30 min in a bath of 45 mM CaCl₂ in hgDMEM, and then the cell-laden hydrogel were maintained in XPAN medium for 24 h before switching to chondrogenic medium (CDM) (Table 3.2) at 5% pO₂. Media exchange was performed twice weekly until the end of the 4-week culture period.

Table 3.2. Composition of the chondrogenic medium.

Chondrogenic Medium (in hgDMEM)	
100 U/mL Penicillin (Gibco, Biosciences)	100 µg/mL Streptomycin (Gibco, Biosciences)
100 µg/mL Sodium pyruvate (Sigma-Aldrich)	40 µg/mL L-proline (Sigma-Aldrich)
50 µg/mL L-ascorbic acid-2-phosphate (Sigma-Aldrich)	4.7 µg/mL Linoleic acid (Sigma-Aldrich)

1.5 mg/mL Bovine serum albumin (BSA; Sigma-Aldrich)	1 X Insulin–Transferrin–Selenium (Sigma-Aldrich)
100 nM Dexamethasone (Sigma-Aldrich)	2.5 µg/mL Amphotericin B (Sigma-Aldrich)
10 ng/mL of human transforming growth factor-β3 (TGF-β3; Peprotech)	

Live/Dead Imaging: Cell viability was assessed via a live/dead assay at day 7 after bioprinting. Briefly, constructs were washed in phenol-free DMEM (pfDMEM; Sigma-Aldrich) followed by incubation in pfDMEM containing 2 µM calcein acetoxymethyl (calcein AM) and 4 µM ethidium homodimer-1 (EthD-1) (both from Bioscience) for 1 h. Samples were washed in pfDMEM before imaging with a Leica SP8 scanning confocal microscope excited at 494 nm and 528 nm, and read at 517 nm and 617 nm.

Biochemical Analysis: The biochemical content of the constructs was analysed after 4 weeks of *in vitro* culture. Constructs were washed in deionised water, frozen, and freeze-dried to obtain the dry weight. Bioprinted constructs were digested using a 3.88 U/mL papain solution in ultra-pure water containing 5 mM L-cysteine–hydrochloride hydrate, 0.1 M sodium acetate, 5 mM methylenediaminetetraacetic acid (EDTA) (all from Sigma–Aldrich). The pH was adjusted to 6.5 using 38% HCl. Samples were incubated in papain solution at 60 °C, rotating at 10 rpm overnight. DNA content was quantified immediately after digestion using the DNA Quantification Kit (Merck). The amount of sulphated glycosaminoglycan (sGAG) was determined using the dimethylmethylene blue dye-binding assay, with a chondroitin sulphate solution (Blyscan, Biocolor Ltd.) for the standards. The pH of the DMMB was adjusted to 1.5 using a 12 N HCl solution. Total collagen content was quantified by determining the hydroxyproline content of the constructs using the dimethylaminobenzaldehyde and chloramine T assay, and calculated assuming a hydroxyproline-to-collagen ratio of 1:7.69 [273].

Histological Analysis: Constructs were fixed in 4% paraformaldehyde (PFA), dehydrated in a graded series of ethanol and xylene baths, embedded in paraffin wax, sectioned at 7

µm using a microtome (Leica Microsystems), and affixed to microscope slides. The sections were stained with haematoxylin and eosin (H&E), alcian blue/aldehyde fuchsin, picrosirius red and alizarin red. Antigen retrieval was carried out by an initial treatment with pronase (3.5 U/mL; Merck) at 37 °C for 25 min, followed by hyaluronidase (4000 units/mL; Sigma-Aldrich) at 37 °C for 25 min for collagen type I and type II. For collagen type X, the antigen retrieval method consisted of an initial treatment with pronase (35 U/mL; Merck) at 37 °C for 5 min, followed by chondroitinase ABC (0.25 U/mL; Sigma-Aldrich) at 37 °C for 45 min. Non-specific sites were blocked using a 10% goat serum and 1% BSA blocking buffer for 1 h at room temperature. Collagen type I (1:400; ab138492; Abcam), type II (1:400; sc52658; Santa Cruz), and type X (1:300; ab49945; Abcam) primary antibodies were incubated overnight at 4 °C, followed by 20 min treatment using a solution of 3% hydrogen peroxide (Sigma-Aldrich). The secondary antibodies for collagen type I (1:250; ab6720; Abcam), type II (1:300; B7151; Sigma-Aldrich) and type X (1:500, ab97228; Abcam) were incubated for 1 h at RT. Samples were then incubated for 45 min with VECTASTAIN Elite ABC before treating them with ImmPACT DAB EqV (both from Vector Labs) at RT. Slides were then imaged using an Aperio ScanScope slide scanner.

Scanning Electron Microscopy (SEM): For SEM imaging, samples were frozen in liquid nitrogen, and immediately dehydrated by lyophilisation at −10 °C overnight. Samples were cut in half and mounted on SEM pin stubs with carbon adhesive discs and coated with gold/palladium for 60 s at a current of 40 mA using a Cressington 208HR sputter coater. Imaging was carried out in a Zeiss ULTRA plus SEM.

Rheological Assessment of Bioinks: The rheological properties of all the bioinks were evaluated using a rheometer (MCR 102, Anton-Paar) equipped with a Peltier element for temperature control. A plate-plate geometry with a diameter of 25 mm (PP25) was used in all the tests. The viscosity as a function of shear rate (0.1 to 1000/s) was conducted at a constant temperature of 16 °C. Then, the gelation kinetics were assessed with a

temperature sweep from 37 °C to 4 °C with a reduction rate of 5 °C/min while maintaining the shear rate at 1/s. Bioinks were kept in a high humidity atmosphere to prevent dehydration from affecting the rheological results.

Degradation Rates of Alginates and Gelatine Elimination Analysis: To determine the degradation rate of the different bioinks cylindrical-shaped constructs of 5 mm diameter and 3 mm height were printed as described above. After crosslinking with CaCl₂, the constructs were then transferred into a 24-well plate, and cultured for 28 days at 37 °C. Media was changed twice every week using hgDMEM. At different time points, samples were washed with deionised water and frozen at –80 °C. The samples were lyophilised to obtain the dry weight. To investigate the presence of gelatine after the incubation of the hydrogels at 37 °C, a hydroxyproline assay was performed as described in the biochemical analysis methods.

Mechanical Testing: To investigate how the different degrees of oxidation and blends influence the mechanical properties of the final gel, constructs were 3D printed as described previously. Mechanical compression tests were performed using a single column Zwick (Zwick, Rowell) with a 10 N load cell in wet conditions using a PBS bath as previously reported [274]. The Young's modulus was defined as the slope of the linear phase of the resulting stress-strain curve during the ramp phase of the compression to 10% strain.

Statistical Analysis: GraphPad Prism software was used to perform all the statistical analysis. Results are displayed as mean ± standard deviation, and were analysed by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or Student's t-test. Significance was determined at $p \leq 0.05$. Sample size number (n) is specified within the respective figure legends.

3.3. Results

3.3.1. Alginate oxidation and characterisation

Partial oxidation of alginate was carried out using sodium periodate (NaIO_4) as an oxidising agent. This chemical reaction leads to the formation of two aldehyde groups, and the cleavage of a carbon-carbon bond, creating an alginate that is hydrolytically labile (Figure 3.1B). By controlling the stoichiometric addition of NaIO_4 , with respect to the alginate content, the sugar monomers in the alginate chains were partially oxidised to different extents (1, 4, 5 and 10%). The ^{13}C NMR spectra of different oxidised alginates displayed an increased peak at 92.2 ppm corresponding to the aldehyde groups (Figure 3.1C) [275]. The degradation profile of the different alginates was examined by maintaining alginate gels in standard cell culture conditions for 28 days (Figure 3.1D; Supporting Figure S3.1A). The dry weight of the unmodified alginate and the 1% partially oxidised gels did not show any mass loss during the 4 weeks in culture, whereas the alginates with a 4% and 5% degree of oxidation had completely degraded after 28 and 14 days, respectively. We were unable to form hydrogels with the 10% oxidised alginates, and thus this condition was not further characterised. The compressive properties of the alginate-based hydrogels decreased as a function of increasing percentage of oxidation (Supporting Figure S3.1B). The elastic modulus reduced from 29.03 ± 1.61 kPa for the unmodified alginate to 9.15 ± 3.60 and 3.68 ± 0.39 kPa for the partial oxidations of 4 and 5%, respectively. As the 4% alginate underwent complete degradation over 4 weeks, a time period within the range typically used for *in vitro* cartilage tissue engineering studies, this condition was selected for further analysis and development. Such a degradation timeframe would also ensure that the majority of alginate material was cleared from a bioprinted tissue before its implantation into the body. From this point onwards, we will refer to the 4% oxidised alginate as ‘OA’.

We next evaluated the chondrogenic capacity of porcine MSCs encapsulated within OA and raw alginate to discard any detrimental effect due to the presence of

aldehyde groups in the polymer backbone. Immediately after hydrogel encapsulation, no major differences in cell behaviour could be observed, with rounded cells observed in both conditions (Figure 3.1E). After 24 h, small clusters of cells started forming in the OA gels, which increased in size after 48 h and remained for the entire period of culture. The DNA content within the OA decreased during the first week of culture, and then remained constant throughout the 28-day culture period. Conversely, the DNA levels in unmodified alginate hydrogels did not change over time. Histological evaluation revealed that cells were distributed throughout the depth of the unmodified alginate hydrogels but appeared more clustered within the OA hydrogels (Figure 3.1G). The strong staining for new tissue deposition within both groups indicated that the presence of aldehyde groups did not affect the chondro-permissiveness of the gels (Figure 3.1G), with robust sGAG and collagen deposition observed in both groups (Supporting Figure S3.2). However, the OA gels had lost their mechanical stability by the end of the culture period, suggesting that their rate of degradation was still too fast for the cell secreted ECM to stabilise the 3D structure.

3.3.2. Bioink characterisation

Having demonstrated the capacity of OA hydrogels to completely dissolve after 4 weeks, we sought to investigate the potential of combining slow (raw alginate) and fast (OA) degrading networks to obtain a more balanced degradation rate suitable for *in vitro* tissue engineering and 3D bioprinting (Figure 3.2A). For the purpose of this study, 5 different hydrogel inks were designed by changing the ratio of raw alginate to OA (Alginate:OA): 100:0; 75:25; 50:50; 25:75; 0:100. The oxidation process greatly reduced the polymer viscosity compared to raw alginate, making it unsuitable for extrusion bioprinting (Figure 3.2D). In order to make the alginate-based inks printable, we incorporated gelatine as a temporary viscosity enhancer to enhance their rheological performance, which supported the development of a continuous filament during extrusion-based bioprinting (Figure 3.2B). This can be attributed to the higher viscosity of the gelatine, which displays a clear

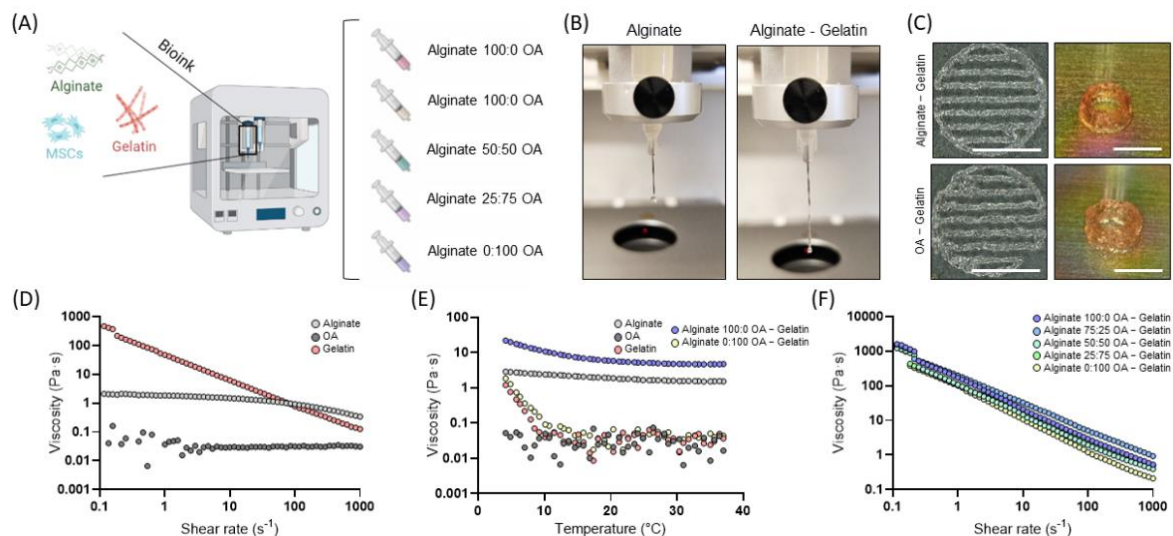


Figure 3.2. Bioink characterisation . (A) Schematic of the experimental plan. Illustration of the bioink composition and different blends investigated. (B) Droplet/Filament formation demonstrating the role of gelatine for printing low viscous inks. (C) Printed hydrogels following grid and tubular patterns. Scale bar is equal to 6 mm. (D) Viscosity of the individual components (polymers) investigated under a shear rate in the range of 0.1 to 1000 s⁻¹ at 16 °C. (E) Viscosity of bioinks and individual components as a function of temperatures changing from 37 to 4 °C. (F) Viscosity of the different bioinks in the presence of shear rate in the range of 0.1 to 1000 s⁻¹ at 16 °C.

shear-thinning behaviour (Figure 3.2D). Due to the thermal-gelation properties, the viscosity of gelatine-containing inks increased when the temperature was decreased from 37 to 4 °C, indicating that temperature can be used to tune the rheological properties of these bioinks (Figure 3.2E). After gelatine gelation at 16 °C, all the bioinks displayed similar rheological properties with a pronounced shear thinning behaviour (Figure 3.2F). For that reason, 16 °C was selected as the optimal temperature to carry out all bioprinting steps. The different gelatine-containing bioinks could be successfully printed into patterns or tubular structures (6 cm Ø and 3 cm height) with a spreading ratio of 1.79 ± 0.17 for the alginate-only bioink (100:0), and 1.93 ± 0.18 for the OA-only bioink (0:100), see Figure 3.2C and Supporting Figure S3.3.

3.3.3. Degradation rate of the blended bioinks

In order to assess the degradation profile of the different bioinks, cylindrical constructs were printed and maintained in culture for a 28-day period. A rapid decrease in dry weight was seen in all the bioinks after 48 h, which can be related to the release of gelatine after the incubation of the constructs at 37 °C (Figure 3.3A). As seen from the decrease in hydroxyproline content in each hydrogel, and the SEM images, most of the gelatine was released after 48 h, leaving behind pores in the alginate gels (Figure 3.3B,C; Supporting Figure S3.4). At longer incubation times, the 100:0 and 75:25 gels displayed minimal additional mass loss. In contrast, the 50:50 and 25:75 gels displayed time-dependent reductions in their mass. Specifically, the 25:75 group lost a further 45% of its dry weight between day 2 and day 28, whereas the 50:50 had lost a further 23% of its mass within the same period. As expected, the only group which underwent a complete mass loss during this time was the 0:100 group.

In terms of their mechanical stability, all the bioinks containing unmodified alginate were able to retain their initial cylindrical shape (measuring the base diameter and height of the construct; Figure 3.3D). We also mechanically characterised the different constructs across the 4-week time period (Figure 3.3E). The initial compressive modulus dramatically decreased in all bioink groups after 7 days. Statistically significant differences in stiffness were observed between the different groups at all time points, with stiffness decreasing as the amount of unmodified alginate was reduced. There was no significant decrease with time in the mechanical behaviour of the 100:0 constructs, which was consistent with the mass loss data. In contrast, the ramp modulus of the 75:25, 50:50 and 25:75 constructs decreased by 25, 47 and 37% over the 28-day period.

3.3.4. Cell viability and chondrogenic differentiation

We next sought to evaluate the capacity of these different bioink blends to support chondrogenesis of encapsulated MSCs over 4 weeks in culture post-bioprinting.

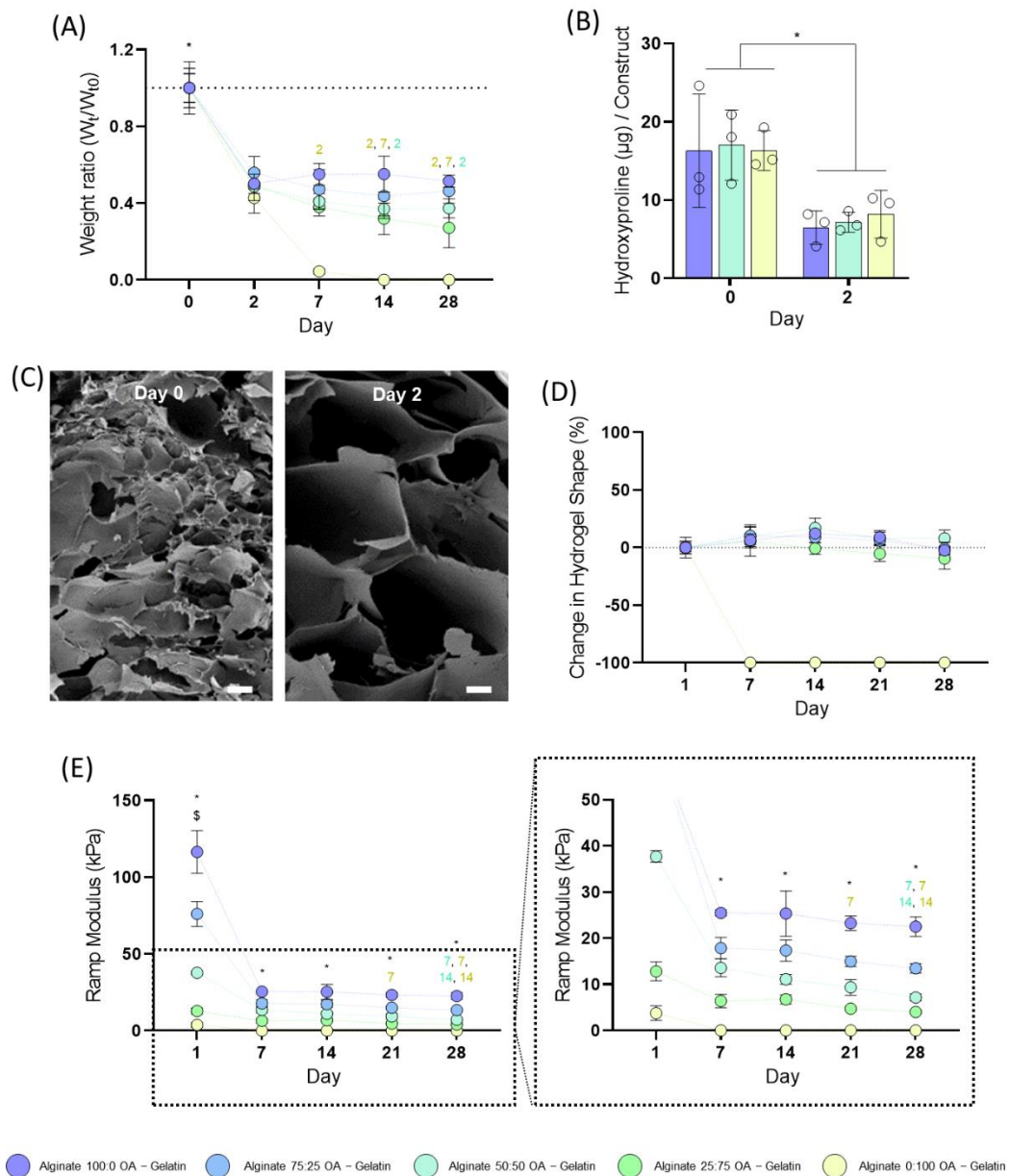


Figure 3.3. Hydrogel characterisation . (A) Dry weight of 3D printed hydrogels at various time points. * different to all time points; 2,7 different to day 2 and day 7 (colour equals condition). (B) Quantification of the hydroxyproline levels in each construct at day 0 and day 2. (C) SEM images on Day 0 and Day 2 revealing the increased porosity after the gelatine removal. The scale bars are equal to 20 μm . (D) Hydrogel shape tracking as a function of time. (E) Mechanical properties of the different bioinks at different time points. * all groups are different; 7, 14 different to day 7 and day 14 (colour equals condition). All error bars denote standard deviation, significance was considered $p \leq 0.05$, $n = 4$; except (B) that $n = 3$.

Live/dead staining after 7 days in culture revealed a highly viable cell population (Figure 3.4A, Supporting Figure S3.5). Since the 0:100 construct had completely lost its shape and degraded within 7 days, no additional cell characterisation was performed on this group. As expected, cells displayed a round morphology within the alginate-based bioinks. In the 25:75 group, MSCs started forming small clusters as the hydrogel degraded. After 4 weeks of culture, alcian blue/aldehyde fuchsin staining demonstrated high levels of sGAG deposition in all bioinks (Figure 3.4B). No differences in DNA

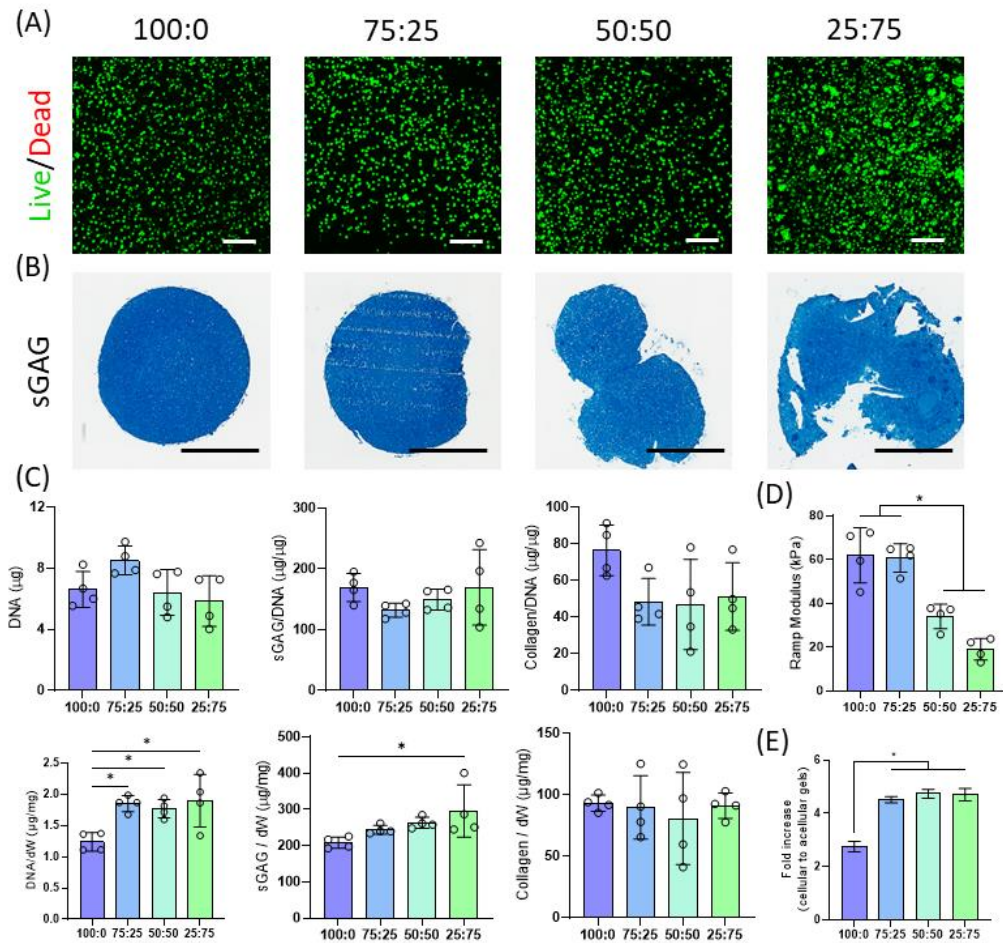


Figure 3.4. Chondrogenic evaluation of the bioinks. (A) Representative images of the live/dead staining at day 7. Green and red indicate live and dead cells, respectively. Scale bars are equal to 200 μm . (B) Alcian blue/aldehyde fuchsin staining for sulphated glycosaminoglycan (sGAG). Scale bars are equal to 2 mm. (C) Quantification of DNA, sGAG and collagen deposition in each construct at day 28. (D) Ramp modulus of the constructs derived from the linear region of compressive strain-stress curves after 28 days in culture. (E) Fold change in the ramp modulus of acellular and cellular hydrogels following 4 weeks of *in vitro* culture. All error bars denote standard deviation, significance was considered $*p \leq 0.05$, $n = 4$.

content were seen across the groups (Figure 3.4C). Similarly, no differences in sGAG and collagen synthesis, when normalised to DNA, were observed across the groups. However, when normalised to the construct dry weight, significantly higher levels of sGAG were observed in the 25:75 group compared to the unmodified alginate bioinks.

Mechanical testing at the end of the 28-day culture period revealed that the stiffness was lower in OA-containing constructs (Figure 3.4D). Compared to the acellular hydrogels at the same time point, the ramp modulus had increased 2.75 and 4.51 times for the 100:0 and 75:25, and 4.73 and 4.70 times for the 50:50 and 25:75, respectively (Figure 3.4E).

Collagen staining further indicated robust chondrogenic differentiation of the encapsulated MSCs in all bioinks, with no calcium deposits observed as evidenced by negative staining for alizarin red (Figure 3.5). Immunohistochemical analysis revealed strong staining for collagen type II, with some type I deposition. Although negligible

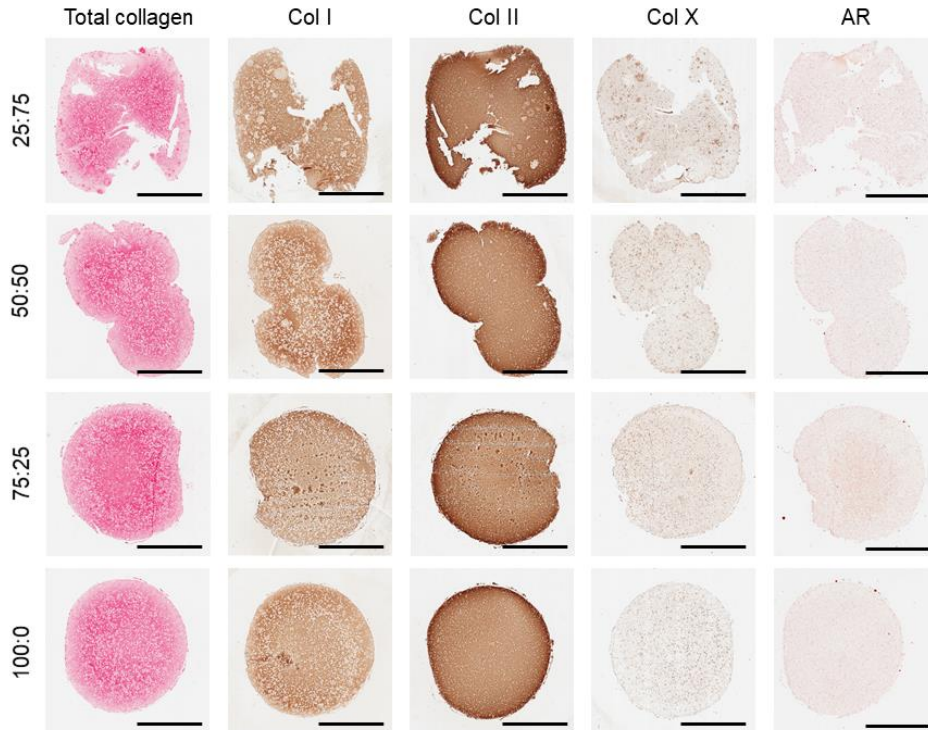


Figure 3.5. Histological evaluation. Picrosirius red staining for total collagen. Immunohistochemical staining for collagen types I, II, and X. Alizarin red (AR) staining for calcium deposits. Scale bars are equal to 2 mm.

staining for collagen type X was observed in the 100:0, 75:25 and 50:50 groups after 4 weeks of culture, small pockets of tissue staining positive for this hypertrophic marker was observed in the 25:75 constructs, which correlated with the formation of cellular clusters.

3.4. Discussion

In this study, we sought to develop bioinks with temporally defined rates of degradation to support the *in vitro* formation of functional cartilaginous tissues. The combination of partially oxidised and unmodified alginate enabled finer control of hydrogel degradation, while maintaining the 3D structure of the printed construct in culture. The bioink 25:75 was softer and underwent faster degradation, resulting in higher levels of cellular clustering. All of the alginate-based bioinks were found to support robust chondrogenesis of MSCs, with no major differences in cellular phenotype observed with changes in the rate of ink degradation. Taken together, these findings support the use of alginate-based bioinks with temporally defined rates of degradation to support the *in vitro* biofabrication of tissues such as articular cartilage, hypertrophic cartilage or meniscus. Designing inks that rapidly degrade upon the establishment of a functional repaired tissue will be integral to the development of successful bioprinting strategies, as it will help to mitigate concerns associated with a negative *in vivo* response (e.g., a foreign body response) to implanted materials.

As expected, the oxidation of the alginate polymer resulted in hydrogels with lower mechanical properties and faster degradation [276-278]. Partial oxidation of alginate using sodium periodate leads to the formation of aldehyde groups in the oxidised monomers, but also reduces the molecular weight of the polymer. Here, partial oxidation above 5% yielded OAs which could not form stable gels (e.g., 10% oxidation), whereas 5% oxidation rates resulted in weak gels that fully dissolved after 1 week (Supporting Figure S3.1), which is consistent with previous studies [277]. For this reason, 4% oxidation was selected for further development since it would completely degrade

relatively quickly (2–4 weeks), providing encapsulated cells with the space to condensate and secrete cartilaginous ECM components. However, the rapid degradation and low mechanical properties of the 4% oxidised alginate led to their physical collapse during *in vitro* culture, causing changes to the initial shape of the constructs and a loss of encapsulated cells (Figure 3.2E–G). This is a problem for 3D bioprinting strategies where maintaining a specific shape is integral to success.

In order to more tightly control the rate of ink degradation, we explored the blending of oxidised and non-oxidised alginates to obtain bioinks with different degradation rates. In many previous studies using alginate as a bioink, the use of a temporary viscosity enhancer such as gelatine or methylcellulose, or the addition of CaCl_2 to partially crosslink the alginate pre-printing, is generally required to enable printing [137, 270, 279–282]. The incorporation of 5% (w/v) gelatine into the alginate blends conferred the bioinks with rheological properties compatible with extrusion bioprinting [128]. All gelatine-containing bioinks displayed comparable rheological properties that permitted the use of similar 3D bioprinting methodologies and parameters, despite the fact that the alginate blends showed very different viscosities in isolation. After placing the constructs in the incubator at 37 °C (which is above the melt temperature for gelatine), the gelatine was gradually released from the hydrogel inks (Figure 3.3A,B). By measuring the dry weight and analysing the hydroxyproline content in the gels, we could determine that most of the gelatine was washed out after 2 days, leaving behind open pores in the alginate gels similar to observations in previous works (Figure 3.3C) [270]. In spite of the gelatine release, the initial 3D printed geometry was maintained throughout the culture period for all constructs containing a fraction of unmodified alginate.

All the different bioinks supported high levels of cell viability and robust chondrogenic differentiation of MSCs. The different alginate blends supported comparable levels of sGAG and collagen deposition, demonstrating that the altered biophysical properties of the OA-containing inks did not negatively impact their capacity

to support robust new tissue formation. The amount of DNA and sGAG (per dry weight of the construct) was higher in the 25:75 group, which can be attributed to the fact the oxidised polymer is more susceptible to hydrolytic scission, thereby decreasing the hydrogel dry weight. The high levels of collagen type II seen across all groups confirm the development of cartilage tissue, which can be linked to the absence of cell-binding motifs forcing cells to acquire a rounded morphology associated with the hyaline cartilage phenotype [1]. Interestingly, collagen type X staining was only detected in the group with larger amounts of OA where cells were allowed to cluster together. The formation of cell clusters is a hallmark of osteoarthritic cartilage formed after cell migration and/or proliferation, which express high levels of collagen type X [283]. The clustering of cells in these OA hydrogels can be linked to the rapid degradation of the gel, which facilitates cell migration and clustering. Hydrogel stiffness is another key factor that has been shown to regulate MSC chondrogenesis and hypertrophy. While previous studies have generally reported that stiffer hydrogel environments commonly enhance the expression of markers associated with chondrocyte hypertrophy [284, 285], it should be recognised that softer hydrogels that facilitate cell-cell interactions will generally accelerate chondrogenesis of MSCs and lead to associated increases in the expression of hypertrophic markers such as type X collagen [286].

The main limitation of this study is the fact that stiffness and degradation are coupled, which means faster degrading hydrogels are softer, increasing the number of variables that are known to influence cellular phenotype within such hydrogel systems. A possible solution to uncouple both degradation and stiffness is the use of binary alginate gels [134, 276, 287]. Using such systems, it is possible to generate hydrogels with comparable stiffness but presenting different degradation rates by combining alginates with different molecular weights and extents of oxidation. While not measured here, it is also likely that the viscoelastic properties of the bioinks (e.g., stress relaxation) also change with the ratio of unmodified alginate to OA, a factor that is also known to regulate chondrogenesis *in vitro* [288]. Future studies will look into developing a library of

alginate-based bioinks with different degradation rates whilst also controlling for other key factors such as hydrogel stiffness and stress relaxation. Recent work has described a promising approach to monitor scaffold degradation during neocartilage generation by covalently binding a fluorophore to the polymer (GelMA) backbone [260]. By doing so, the loss of fluorescence from the scaffolds was correlated with their degradability, which would benefit the high throughput monitoring of tissue engineering therapies.

3.5. Conclusion

In summary, a strategy was developed to obtain bioinks that can act as temporal support structures for tissue engineering and bioprinting applications. Here, the partial oxidation of the alginate (4%) permitted its complete degradation after 4 weeks in culture. However, this rapid degradation led to a complete loss of the initial 3D structure, which was overcome by combining oxidised and unmodified alginates. The addition of 5% (w/v) gelatine to the alginate-based bioinks greatly improved their rheological behaviour, making them suitable for extrusion bioprinting. These tuneable bioinks were capable of structurally supporting the chondrogenic differentiation of MSCs for a period of 28 days without collapse or significant changes in the printed shape. In the context of tissue engineering and bioprinting, these temporal bioinks show significant potential for the *in vitro* biofabrication of tissues that are rapidly cleared of supporting exogenous biomaterials, ultimately resulting in ‘scaffold-free’ bioprinted tissues which can be more easily integrated *in vivo*.

Chapter 4: Bioprinting of structurally organised meniscal tissue within anisotropic melt electrowritten scaffolds

4.1. Introduction

Injuries to the meniscus commonly initiate degenerative changes to the articular cartilage and other joint tissues, which eventually leads to the development of osteoarthritis [26, 63]. The main function of the meniscus is to maintain stability and distribute the load within the knee joint. During normal daily activities, the meniscus converts vertical axial forces within the knee joint to hoop stresses in the tissue which are borne predominately by circumferentially arranged collagen fibres. In spite of their extraordinary mechanical properties, menisci are prone to lesions, often referred to as meniscal tears [289, 290]. Due in part to its limited vascularity, which is confined to the outer region of tissue, the innate regenerative capability of the meniscus is poor. For severe injuries, current treatments such as partial or total meniscectomy only provide short-term relief. Tissue engineering strategies represent a promising alternative to meniscectomy and allografts [37, 291-293], with biomaterial scaffolds produced from collagen or polyurethane already in clinical use [35, 294]. Unfortunately, these biomaterial-based solutions currently in clinical use do not result in functional meniscus regeneration. This motivates the use of new and emerging biofabrication and 3D bioprinting strategies for the development of engineered tissues mimicking the structure, composition, and biomechanics of native meniscus [52, 295].

The meniscus is an intricately organised structure characterised by its significant anisotropy and compression-tension non-linearity that is integral to the biomechanical function of this soft tissue [296]. The composition of this semilunar fibrocartilaginous

tissue changes from its inner zone to its outer zones, which contain radially and circumferentially aligned collagen fibres that define the anisotropic mechanical properties of the tissue [23, 297]. A small number of studies have successfully engineered fibrocartilaginous tissues mimicking certain architectural features of the native meniscus. For example, self-assembly tissue engineering strategies have shown that the contractile forces generated by cells within ring-shaped, non-adherent moulds induced circumferential collagen alignment, while the application of biochemical stimuli has been shown to support the generation of more biomimetic neomenisci constructs [178, 298]. Anisotropic porous scaffolds can also be used to direct cell alignment, strategies which have also been applied to meniscus-shaped scaffolds [299, 300]. Aligned electrospun scaffolds have also been investigated as a template for cells to direct tissue growth, supporting the engineering of anisotropic fibrocartilaginous constructs [161]. However, the biofabrication of regionally defined implants mimicking specific anatomical sizes and shapes may be challenging with the aforementioned techniques, motivating the use of convergent 3D bioprinting strategies to address these limitations while also providing the potential to develop patient-specific implants [301].

Advances in 3D bioprinting are already supporting the engineering of meniscal-like constructs, from the development of bioinks to support a meniscal cell phenotype [130, 140, 155, 302], to the use of fibre reinforcement strategies to print constructs with bulk mechanical properties similar to the native meniscus or to help maintain a uniform distribution of cells and bioactive factors encapsulated within a soft hydrogel [2, 142, 146, 164, 303]. While existing bioprinting strategies have had some success in mimicking certain compositional or structural features of the meniscus [3, 8, 293, 304], engineering tissues with an anisotropic collagen architecture which mimics that of the native tissue remains a challenge. We have previously shown that zonally stratified articular cartilage can be engineered by inkjet bioprinting mesenchymal stem/stromal cells (MSCs) into guiding polycaprolactone (PCL) scaffolds produced using either fused deposition modelling (FDM) or melt electrowriting (MEW) [221, 244, 245]. PCL is a synthetic

thermoplastic polymer that is commonly used in the field of 3D (bio)printing due to its low melting temperature, biocompatibility, relatively slow degradation rate and mechanical properties [188]. We demonstrated that these PCL structures acted as boundary conditions to direct neotissue growth and organisation following inkjet bioprinting. MEW is particularly an attractive biofabrication technique for the engineering of soft tissues, as it enables the printing of highly porous scaffolds with fibre diameters orders of magnitude smaller than that possible using conventional FDM strategies [193, 195, 244, 305-307]. This in turn enables the printing of scaffold structures that are soft in compression and relatively stiff in tension, similar to mechanical properties provided by the collagen network in native articular cartilage and meniscus [308-312]. Built on these earlier findings, the aim of this study was to establish the optimum additive manufacturing technique (either FDM or MEW) which can be used to print guiding PCL structures to support the engineering of meniscal grafts with anisotropic collagen networks. Using inkjet bioprinting as part of a multiple-tool biofabrication strategy, defined numbers of cells were deposited into 3D printed scaffolds consisting of arrays of guiding PCL microchambers. By altering the aspect ratio of these PCL microchambers, it was possible to alter not only the anisotropic mechanical properties of the cell-laden grafts, but also the architecture of the collagen network laid down by the jetted cells. Finally, as a proof of concept, we fabricated spatially inhomogeneous MEW scaffolds with specific regions mimicking aspects of the inner and outer zones of the native meniscus.

4.2. Material and methods

Scaffold Fabrication

Fused Deposition Modelling (FDM): FDM scaffolds with a fibre diameter of 150 μm were fabricated using the thermopolymer printhead of a 3D Discovery (RegenHU). Porous PCL (50,000 MW, CAPA 6500D, Perstorp) scaffolds were fabricated with three different architectures as defined by their aspect ratio (AR): 0.8 x 0.8 mm (AR 1:1), 0.4

x 1.6 mm (AR 1:4) and 0.2 x 3.2 mm (AR 1:16). Spacings are defined as the distance between fibres. All printing parameters are described in Table 4.1.

Table 4.1. Machine parameters for FDM

Nozzle	Pressure	Printing speed	Extrusion speed	Temperature	Layer thickness
30G	0.1 MPa	6 mm/s	10 revs/m	Tank: 85°C Needle: 83°C	0.1 mm

Melt Electrowriting (MEW): MEW scaffolds with a fibre diameter of 15 µm were fabricated on a custom MEW printer built in-house [313]. Briefly, PCL (50,000 MW, CAPA 6500D, Perstorp) was melted at a temperature of 80 °C around the syringe, and 85 °C around the nozzle (21G). To extrude the polymer a pressure of 0.6 Bar with a high voltage of 6 kV was applied at a distance of 6 mm from the grounded aluminium collector plate. Fibres were subsequently deposited with predesigned architectures similar to FDM scaffolds, with AR 1:1, AR 1:4 and AR 1:16. Larger scaffolds with spatially distinct architectures (Region A: 0.8mm x 0.8mm; Region B: 0.2mm x 3.2mm) were also manufactured by MEW. To this end, the fibre spacing in the x-direction, either 0.8mm or 0.2 mm, were consecutively printed by changing the spacing during the fabrication process. In region B, fibres were printed in the y-direction in such a way that the fibres will only cross the entire scaffold every 3.2 mm, but maintaining a 0.8mm spacing in region A, thus creating two spatially distinct architectures (see Figure 4.7).

Isolation and Expansion of Mesenchymal Stem/Stromal Cells: Bone marrow-derived MSCs (bMSCs) were obtained from the femur of a pig supplied by a local abattoir (male, approximately 4 months old). The bone marrow was collected under sterile conditions from the femoral shaft, and diluted with expansion medium (XPAN), consisting of hgDMEM supplemented with 10% v/v FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (all Gibco, Biosciences). After obtaining a homogenous suspension using a 16G needle and filtering through a 40-µm nylon mesh,

cells were counted using trypan blue containing 3% acetic acid before plating 23×10^6 cells into T175 flasks for expansion. All expansion was performed at 5% pO₂, and using XPAN medium supplemented with 5 ng/ml FGF2 (Peprotech) seeding at a density of 875,000 cells per T175 flask. Chondrogenic, osteogenic and adipogenic differentiation assays were used to assess the tripotentiality of isolated MSCs. Cells were used at the end of passage 3 for all experiments.

Oxidised Alginate Preparation (OA): The alginate oxidation was performed as previously described [271]. Briefly, 1 g of alginate (MVG, Pronova Biopolymers) was dissolved in 90 mL of deionised water overnight at RT, and mixed with 10 mL of sodium periodate (Honeywell) to achieve a theoretical alginate oxidation of 4% under stirring in the dark at room temperature for 24 h (based on the molecular weight of the repeat unit, MW 198 g/mol). The oxidised alginate was then purified by dialysis against deionised water for 3 days (MWCO 3500 Dalton; Fischer), sterile-filtered through a 0.22 µm filter, and lyophilised.

Inkjet Bioprinting: Inkjet bioprinting technology (3D Discovery, RegenHU) was used to deposit a cell-alginate bioink (30×10^6 cells/mL in 3% (w/v) oxidised alginate). A single droplet was deposited into each well to yield 20×10^3 cells/microchamber for the FDM scaffolds (valve opening time of 650 µs, which yield droplets of 0.666 µL), or 17×10^3 cells/microchamber for the MEW scaffolds (valve opening time of 600 µs, which yield droplets of 0.566 µL) in order to obtain the same seeding number per scaffold. Right after the bioink deposition, a 45 mM CaCl₂ solution in hgDMEM was added to crosslink the alginate for 5 min. The crosslinking solution was then removed and replaced by XPAN medium. After a period of 24 h, all constructs were switched to chondrogenic differentiation medium (CDM), which consisted of hgDMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (both Gibco), 100 µg/mL sodium pyruvate, 40 µg/mL L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 4.7 µg/ml linoleic acid, 1.5 mg/ml bovine serum albumin, 1× Insulin–Transferrin–Selenium (ITS), 100 nM

dexamethasone, 2.5 µg/ml Amphotericin B (all from Sigma) and 10 ng/ml of human transforming growth factor-β3 (TGF-β3) (Peprotech). Throughout the culture, the constructs were maintained at 5% pO₂.

Biochemical Analysis: The biochemical content of the constructs was analysed after 4 weeks of *in vitro* culture. Constructs were washed in deionised water, frozen, and lyophilised to obtain the dry weight. Each construct was digested with papain (3.88 U/mL) in ultra-pure water containing 0.1 M sodium acetate, 5 mM L-cysteine–hydrochloride hydrate, 5 mM methylenediaminetetraacetic acid (EDTA) (all from Sigma–Aldrich). The pH was adjusted to 6.5 using 38% HCl. Samples were incubated in papain solution at 60 °C, rotating at 10 rpm for 18 h. Immediately after digestion, the DNA content was quantified using the Hoechst 33258 dye assay with calf thymus DNA as standard (Merck) reading at 360 nm excitation and 460 nm emission. The amount of sulphated glycosaminoglycan (sGAG) was quantified using the 1,9-dimethylmethylene blue (DMMB) dye-binding assay, with a chondroitin sulphate solution as standard (Blyscan) [314]. To exclude any background absorbance from the alginate, the pH of the DMMB was adjusted to 1.5. The 530/590 absorbance ratio was used to generate the standard curve and determine the sGAG concentration in the digested samples. Total collagen content was determined by measuring the hydroxyproline content using the dimethylaminobenzaldehyde and chloramine T assay, and a hydroxyproline/collagen ratio of 1:7.69 [315]. Briefly, samples were mixed with 100 µL of 38% HCl, and incubated at 100 °C for 18 h. After cooling, samples were centrifuge at 5000 g for 5 min, and let to dry at 50 °C for 48 h. Dried samples were then dissolved in 200 µL ultra-pure water. 2.82 % (w/v) chloramine T solution was added and incubated for 20 min at room temperature in the dark, before the addition of a 50 % (w/v) 4-(Dimethylamino) benzaldehyde solution (both Sigma) at 60 °C for 20 min. Hydroxyproline levels were estimated from the standard curve at a wavelength of 570 nm.

Histological Analysis: Constructs were fixed in 4% (w/v) paraformaldehyde, dehydrated in a graded series of ethanol and xylene baths, embedded in paraffin wax, sectioned at 7 μm using a microtome (Leica Microsystems), and affixed to microscope slides. The sections were stained with haematoxylin and eosin (H&E), alcian blue, picrosirius red and alizarin red. For immunohistochemistry antigen retrieval was carried out by an initial treatment with pronase (3.5 U/ml; Merck) at 37 °C for 25 min, followed by hyaluronidase (4000 units/ml; Sigma-Aldrich) at 37 °C for 25 min for collagen type I and type II. For collagen type X, the antigen retrieval method consisted of an initial treatment with pronase (35 U/mL; Merck) at 37 °C for 5 min, followed by chondroitinase ABC (0.25 U/mL; Sigma-Aldrich) at 37 °C for 45 min. Non-specific sites were blocked using a 10% goat serum and 1% BSA blocking buffer for 1 h at room temperature. Collagen type I (1:400; ab138492; Abcam), type II (1:400; sc52658; Santa Cruz), and type X (1:300; ab49945; Abcam) primary antibodies were incubated overnight at 4 °C, followed by 20 min treatment using a solution of 3% hydrogen peroxide (Sigma-Aldrich). The secondary antibodies for collagen type I (1:250; ab6720; Abcam), type II (1:300; B7151; Sigma-Aldrich) and type X (1:500, ab97228; Abcam) were incubated for 1 h at RT. Samples were then incubated for 45 min with VECTASTAIN Elite ABC before treating them with ImmPACT DAB EqV (both from Vector Labs) at RT. Slides were then imaged using an Aperio ScanScope slide scanner, while sections stained with picrosirius red were imaged using polarised light microscopy. Analysis on the fibre orientation and coherency was carried out using the OrientationJ plugin in ImageJ software [316]. The percentage of area stained DAB positive was determined as previously described to obtain the percentage area positive for a specific type of collagen [317].

Scanning Electron Microscopy: For SEM imaging, samples were frozen in liquid nitrogen, and immediately dehydrated by lyophilisation at -10 °C overnight. Samples were cut in half and mounted on SEM pin stubs with carbon adhesive discs and coated with gold/palladium for 60 s at a current of 40 mA using a Cressington 208HR sputter coater. Imaging was carried out in a Zeiss ULTRA plus SEM.

Rheological Assessment of the Bioinks: The rheological properties of the alginate-based bioinks were evaluated using a rheometer (MCR 102, Anton-Paar) equipped with a Peltier element for temperature control. A plate-plate geometry with a diameter of 25 mm (PP25) was used in all the tests. The viscosity as a function of shear rate (0.1 to 1000 /s) was conducted at a constant temperature of 21 °C.

Mechanical Testing: To investigate the mechanical properties of the engineered tissues the different groups were tested using a single column Zwick (Zwick, Rowell) with a 10 N load cell. Unconfined compression tests were carried out in a PBS bath as previously described [274]. The Young's modulus was defined as the slope of the linear phase of the resulting stress-strain curve during the ramp phase of the compression to 10% strain. Uniaxial tensile tests were performed into rectangular section of dimensions 5 by 30 mm as previously described [195]. The elastic modulus was calculated from the stress-strain curves in the linear region. Yield stress and strain values were determined from the intersection of a 0.2% offset line.

RNA Isolation and Quantitative Real-Time PCR: 4 weeks after *in vitro* culture samples were washed in PBS before snap freezing them until further process. RNA was isolated via Trizol method (Sigma-Aldrich) followed by chloroform extraction as per manufacturer's instructions. RNA was re-suspended in RNase free water, snap frozen in liquid nitrogen and stored at -80°C. Polymerase chain reaction (PCR) with a high-capacity cDNA reverse transcription kit (Thermofisher) was conducted to transcribe 500 ng of RNA from each sample into cDNA. The levels of gene expression were measured with real-time PCR (Applied Biosystems) using SYBR green master mix (Applied Biosystems) and porcine specific primers (Table 4.2). Relative expression was obtained by $2^{-\Delta\Delta C_t}$ method and normalised to the arithmetic average of 2 housekeeping genes 18S and B2M [318].

Table 4.2. List of specific primers for real-time PCR.

Gene name	Gene full name	Forward/Reverse
B2M	beta-2-microglobulin	F:5' ACTGAGTTCACTCCTAACG 3' R:5' TGCAGCATCTTCATAATCTC 3'
18S	ribosomal protein S18	F:5' CAACACCACATGAGCATATC 3' R:5' AGAAGTTCCAGCACATTTTG 3'
ACAN	Aggrecan	F:5' CACCCCATGCAATTTGAG 3' R:5' AGATCATCACACACAGTC 3'
SOX9	SRY-box 9	F:5' GACTGCTGAATGAGAGCGAGA 3' R:5' GAAGATGGCGTTGGGAGAGAT 3'
COL1A1	collagen type 1 alpha 1 chain	F:5' TAGACATGTTTCAGCTTTGTG 3' R:5' GTGGGATGTCTTCTTCTTG 3'
COL2A1	collagen type II alpha 1 chain	F:5' CGACGACATAATCTGTGAAG 3' R:5' TCCTTTGGGTCCTACAATATC 3'
COL10A1	collagen typeX alpha 1 chain	F:5' CCAACATCCAGAATCCATC 3' R:5' GTAGGTGTTTGGTATTGCTC 3'
COMP	cartilage oligomeric matrix protein	F:5' AAGGACAGTGATGGTGATG 3' R:5' ATCCCTCTGATCTGCATTG 3'

Statistical Analysis: Statistical analysis was performed using GraphPad Prism software. Statistical differences were determined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or Student's t-test where appropriate. Results are displayed as mean $\pm$ standard deviation. Significance was accepted at a level of $p \leq 0.05$. Sample size (n) is indicated within the corresponding figure legends.

4.3. Results

4.3.1. Integrating 3D printing of polymeric scaffolds and inkjet bioprinting of cell-laden bioinks

To investigate the effect of scaffold architecture on tissue organisation, we first designed three scaffolds consisting of microchambers with different aspect ratios (AR): 0.8 x 0.8 mm (AR 1:1), 0.4 x 1.6 mm (AR 1:4) and 0.2 x 3.2 mm (AR 1:16). We previously demonstrated that microchambers 0.8 x 0.8 mm in size supported condensation of jetted cells and the development of organised cartilaginous tissue [221], hence here we sought to vary the AR of these microchambers while keeping the area constant in all groups (0.64 mm²). The total height of the scaffolds was 1 mm. Two different additive manufacturing technologies, FDM and MEW, were used to produce these polymeric scaffolds, with fibre diameters of 150 μ m and 15 μ m, respectively (Figure 4.1A). In order to control the spatial

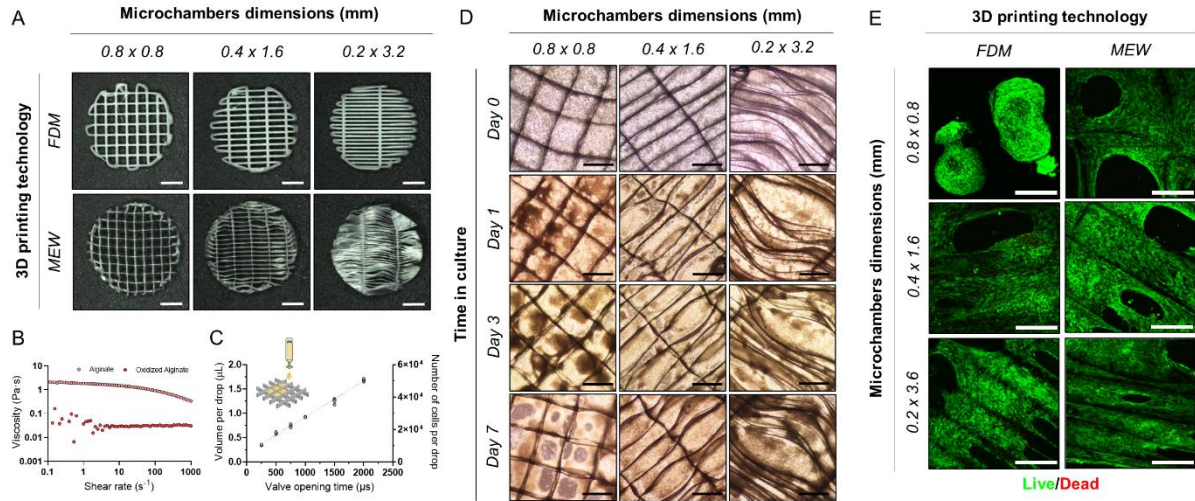


Figure 4.1. Inkjet bioprinting within PCL scaffolds of different architectures. (A) Macroscopic images of the different PCL architectures. Scale bar is equal to 2 mm. (B) Viscosity of unmodified and oxidised alginate in the presence of a shear rate in the range of 0.1 to 1000 s⁻¹. (C) Droplet volume and cell number (30x10⁶ cells/mL) as a function of valve opening time. (D) Phase contrast images of the cell condensation process within the MEW scaffolds after the bioprinting process to day 7 in culture. Scale bar is 800 μ m. (E) Live/Dead images after 7 days in culture. Scale bar is 400 μ m.

deposition of cells within the scaffolds, we required a bioink compatible with inkjet bioprinting that could temporally support the spatial patterning of defined numbers of cells into each microchamber of the scaffold, but which would also rapidly degrade to allow for cellular condensation and the self-organisation of the tissue directed by the differing architectures of the scaffold microchambers. Oxidised alginate (OA) was selected as the biomaterial ink that best fitted our requirements as it can be modified to undergo rapid degradation while simultaneously displaying a viscosity low enough for inkjet bioprinting as previously characterised (Figure 4.1B) [319]. Inkjet bioprinting allowed for the precise control over the droplet volume by changing the opening time of the valve (Figure 4.1C). Since the volume in each microchamber was $\sim 0.64 \text{ mm}^3$, this was identified as the optimal volume of cell-laden bioink to deposit into each microchamber to avoid overfilling of the scaffold. After the inkjet deposition of the cells, the alginate was crosslinked using a bath of CaCl_2 , providing the cells with a temporal structural support within the PCL scaffolds (Figure 4.1D). The rapid degradation of the alginate allowed the condensation of the jetted cells, which rearranged following the architecture of the microchamber. Live/dead imaging indicated excellent cell viability regardless of the 3D printing technology (MEW or FDM) used for the fabrication of the microchamber scaffolds, demonstrating the success of the bioprinting strategy (Figure 4.1E, Supporting Figure S4.1).

4.3.2. Chondrogenesis of MSCs following inkjet bioprinting into polymeric scaffolds

To investigate the effect of scaffold architecture and fibre size on cellular response, we further cultured the cell-laden scaffolds with different aspect ratios in chondrogenic media for 4 weeks. By the end of the culture period, no dramatic differences in DNA levels were observed across the different scaffold groups, with levels comparable to what would be expected based on the initial cell seeding values (Figure 4.2B) (1 million cells were deposited in each scaffold, what corresponds to approximately $7.7 \text{ }\mu\text{g}$ of DNA per

scaffold [320]). DNA levels in the MEW 0.2 x 3.2 mm construct were significantly higher than the corresponding FDM scaffold. Alcian blue staining revealed robust sGAG deposition within all scaffold groups (Figure 4.2A). Significantly higher levels of sGAG synthesis were observed in the MEW 0.8 x 0.8 mm constructs compared to the corresponding FDM scaffold (Figure 4.2C, Supporting Figure 4.2); otherwise sGAG levels were comparable across the different groups. Collagen synthesis was lowest in scaffolds with the longest aspect ratio (0.2 x 3.2 mm), regardless of the fabrication

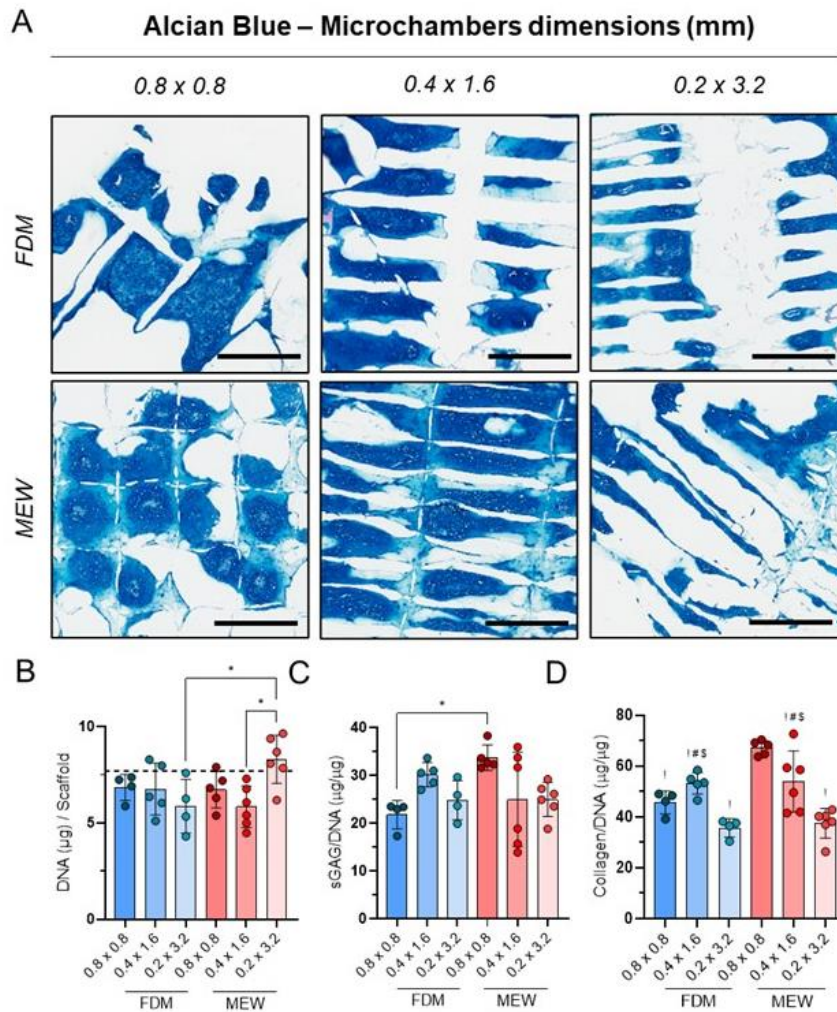


Figure 4.2. Histological and biochemical analysis following 4 weeks of *in vitro* culture. (A) Histological staining for sGAG. Scale bar is equal to 1 mm. (B) DNA content per construct (8 mm Ø). Dashed line corresponds to the initial seeding of 1×10^6 cells. (C) sGAG/DNA. (D) Collagen/DNA. * = significance, ! = significance to MEW 0.8 x 0.8, \$ = significance to FDM 0.2 x 3.2, # = significance to 0.2 x 3.2 MEW. All error bars denote standard deviation, significance was considered $p \leq 0.05$, $n \geq 4$.

approach. The highest levels of collagen synthesis was observed in the MEW 0.8 x 0.8 mm constructs (Figure 4.2D). Collectively these results indicate that both scaffold geometry and the fabrication approach (MEW or FDM) impact the absolute levels of cartilage extracellular matrix deposition in this system, but that all scaffold structures support chondrogenesis of MSCs.

4.3.3. Scaffold architecture dictates tissue orientation

After 4 weeks of *in vitro* culture, MSCs deposited a neotissue directed by the physical boundaries provided by the different scaffold microchamber designs. Picrosirius red staining, which stains for total collagens, demonstrated robust collagen deposition, with polarised light images revealing preferential collagen alignment in the 0.4 x 1.6 and 0.2 x 3.2 mm groups (Figure 4.3). No preferred collagen directionality was observed in the 0.8 x 0.8 mm constructs (Figure 4.3). On the contrary, collagen deposition in the longer aspect ratio constructs preferentially aligned with the long axis of the microchamber walls, which was most evident in the 0.2 x 3.2 mm groups. Fibre coherency (which is a

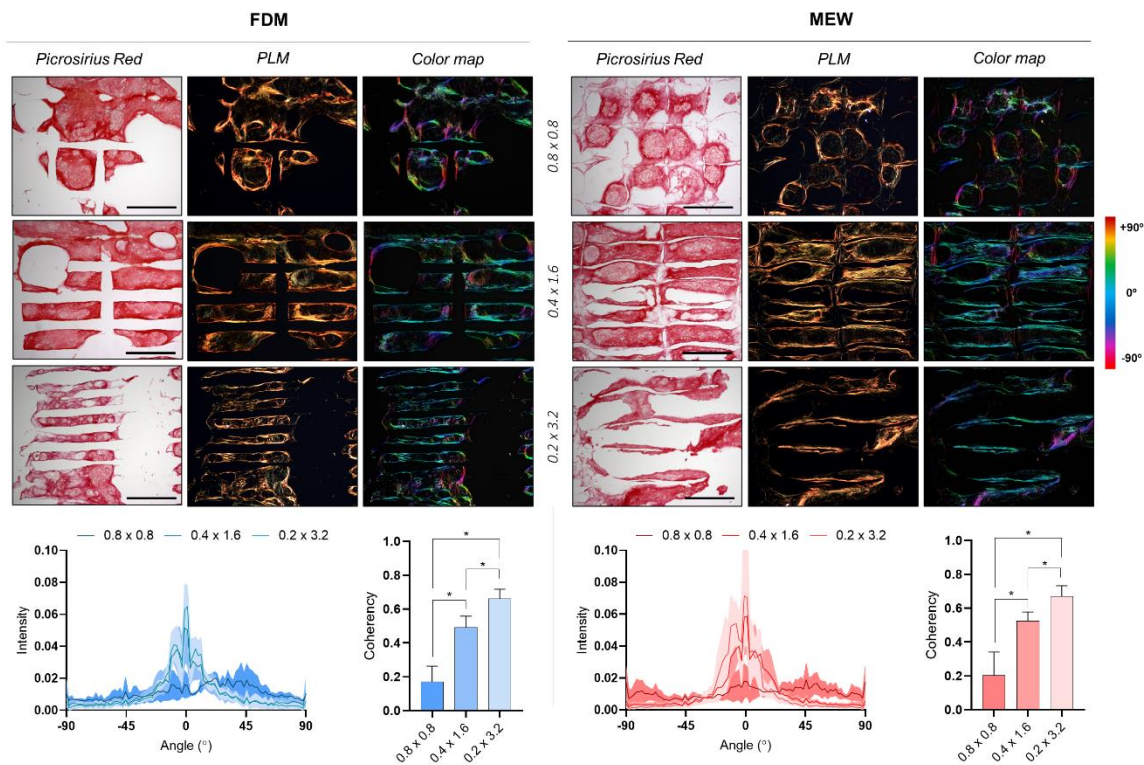


Figure 4.3. Collagen fibre orientations and biomechanical analysis for different scaffold architectures following 4 weeks of *in vitro* culture. Picrosirius red, polarised light (PLM) and colour map imaging of the collagen fibre distributions. Scale bar is equal to 1 mm. Here, cyan/blue colours denote fibres oriented at 0 degrees while purple/red denote fibres oriented at 90 degrees. Quantification of the fibre orientation. Quantification of the fibre coherency, where values approaching to 1 indicating that fibres are aligned towards a preferential direction. All error bars denote standard deviation, significance was considered $*p \leq 0.05$, $n = 6$.

measure of the collagen fibre dispersion) further indicated an increase in overall alignment with increases in the microchamber aspect ratio. However, no major differences were seen between MEW and FDM scaffolds in terms of their capacity to direct tissue organisation. Collectively, these results indicate the potential of using specific scaffold geometries to drive the organisation of engineered tissues.

Finally, the different constructs were tested in uniaxial compression to investigate the biomechanics of the neotissues. As expected, the FDM constructs were relatively stiff, with a ramp modulus between 5 and 10 MPa, which is an order of magnitude above the values for human meniscus (Figure 4.4A, Supporting Figure 4.3) [321]. However, the

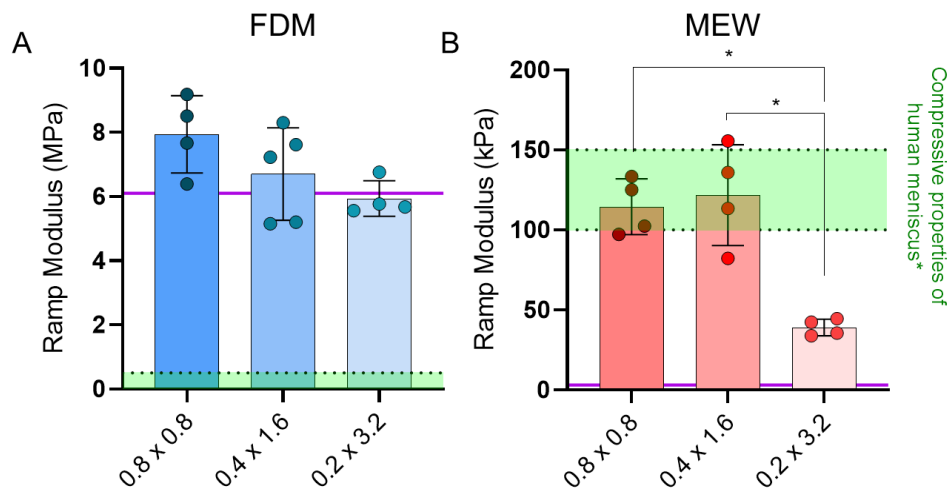


Figure 4.4. Compressive properties following 4 weeks of *in vitro* culture. Ramp modulus of the different constructs following 4 weeks of *in vitro* culture; (A) FDM and (B) MEW PCL scaffolds. Green region denotes native meniscus properties [297]. Purple line denotes average empty scaffold properties. All error bars denote standard deviation, significance was considered $*p \leq 0.05$, $n = 4$.

stiffness of the constructs generated within the MEW scaffolds (between 100 and 150 kPa) after the 4-week culture period was of a similar order of magnitude to the native tissue, but dramatically stiffer than the empty MEW scaffolds (~4 kPa). Collectively these results demonstrate the potential of the MEW technique for the engineering of biomimetic soft tissue constructs (Figure 4.4B).

4.3.4. Tissue engineering of anisotropic meniscal grafts

Having demonstrated that the MEW scaffolds supported (i) chondrogenesis of MSCs, (ii) organised tissue deposition, and (iii) the development of an engineered tissue with compressive mechanical properties comparable to native meniscal tissue, we next sought

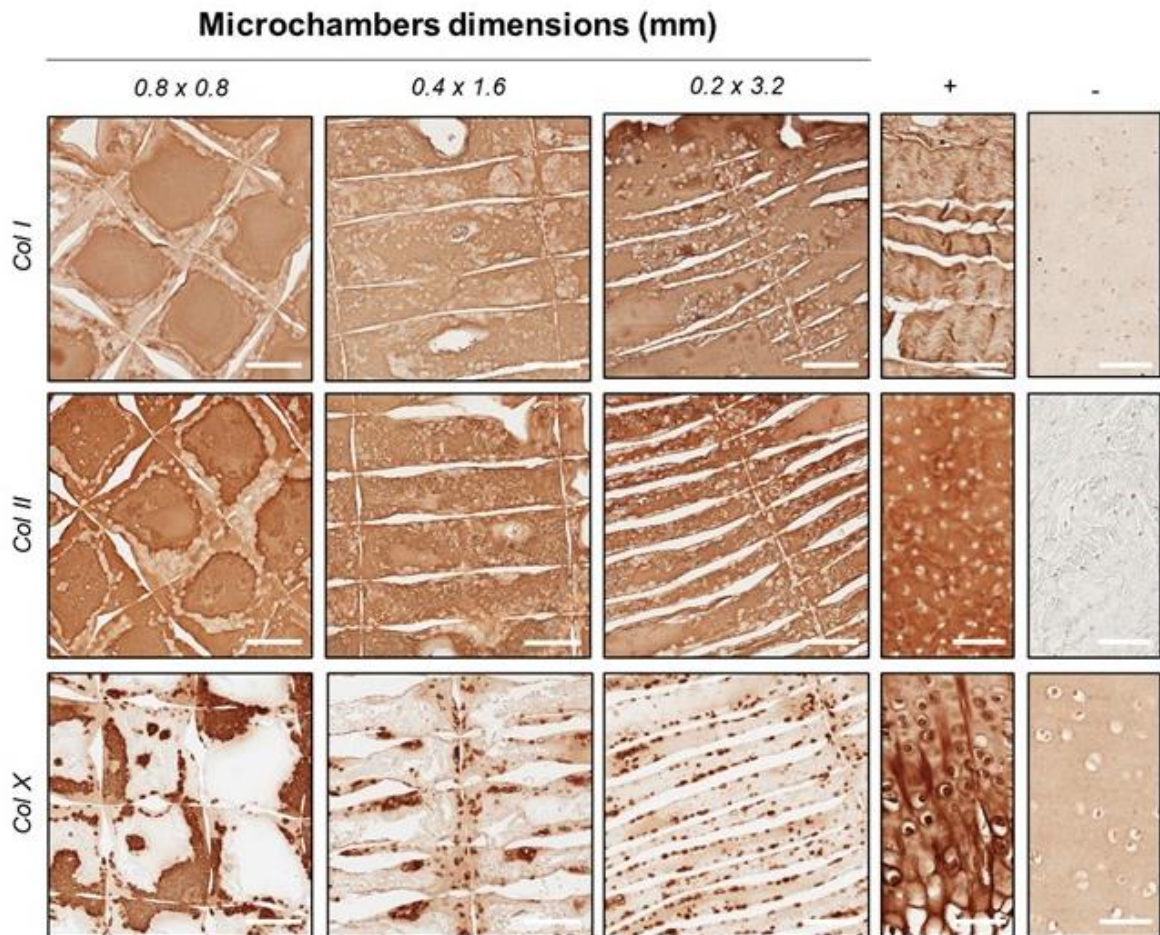


Figure 4.5. Histological evaluation after 8 weeks in culture. Immunohistochemical staining for collagen type I (Col I), collagen type II (Col II) and collagen type X (Col X) deposition. Scale bar is equal to 400 μ m for the engineered tissues and 100 μ m for the immunohistochemical controls.

to better characterise the tissue phenotype supported by these MEW scaffolds and to undertake a longer-term *in vitro* culture period in an attempt to engineer meniscal grafts with anisotropic tensile mechanical properties. qPCR was undertaken after 4 weeks of *in vitro* culture to first assess how MEW scaffold architecture regulated cellular phenotype. No significant differences were observed in the expression of COL1A1, COL2A1, COL10A1, ACAN and COMP as a function of MEW scaffold architecture (Supporting Figure 4.4). Higher expression for SOX9 was seen in the 0.2 x 3.2 mm constructs compared to the 0.8 x 0.8 mm. After 8 weeks *in vitro*, strong staining for collagen types I and II was observed in all MEW scaffold groups (Figure 4.5). Larger regions of tissues forming within the 0.8 x 0.8 mm MEW scaffolds stained intensely for collagen type X staining, suggesting that the microchamber aspect ratio is also a regulator of a hypertrophic cartilage phenotype. Quantification of these stained sections revealed that the 0.2 x 3.2 mm architecture supported overall higher levels of collagen type I deposition, but lower deposition of collagen type II compared to other MEW architectures (Supporting Figure 4.7). Overall, these results suggest that while the geometry of the microchamber has a potent influence on neotissue organisation, their influence on cellular phenotype is more subtle.

We next assessed the anisotropic tensile mechanical properties after 8 weeks of *in vitro* culture by testing the engineered tissues parallel to the long (L) and short (S) axis of the microchambers (Figure 4.6). As expected, the 0.8 x 0.8 mm MEW scaffolds did not support the development of an engineered construct with anisotropic mechanical properties (Figure 4.6A, Supporting Figure 4.5). By changing the aspect ratio of the microchambers, the tensile modulus increased from 1.18 MPa in the 0.8 x 0.8 mm MEW scaffolds to 2.15 and 5.44 MPa, respectively, for the 0.4 x 1.6 and 0.2 x 3.2 mm constructs (parallel to the long axis). At the same time the modulus parallel to the short axis reduced to 0.79 and 0.37 MPa, respectively, for the 0.4 x 1.6 and 0.2 x 3.2 mm groups. Compared to the empty scaffolds, an increase in tensile stiffness was observed with time in culture for all groups (Supporting Figure 4.6). The yield stress was significantly higher parallel

the long axis of the 0.4 x 1.6 and 0.2 x 3.2 mm constructs, while no major differences were seen in the yield strain (Figure 4.6 B,C).

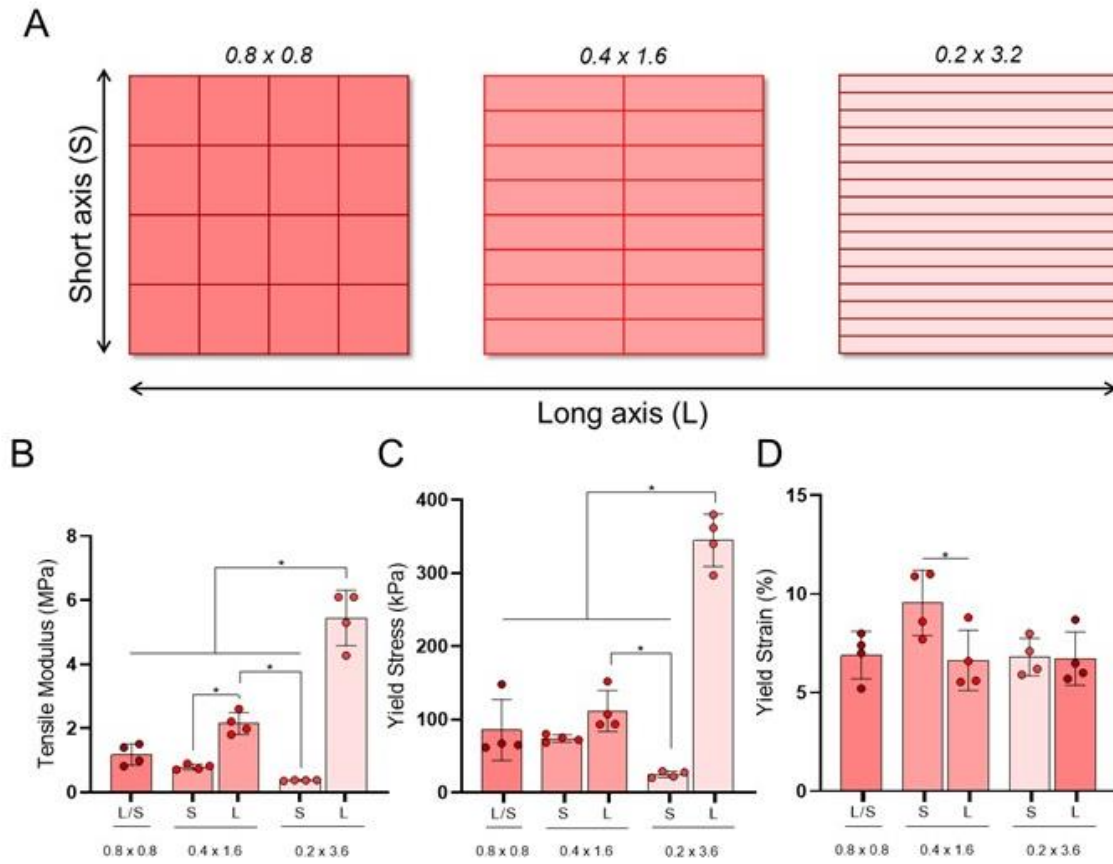


Figure 4.6. Mechanical evaluation after 8 weeks. Tensile properties of the different architectures following 8 weeks of *in vitro* culture. “L” denotes long axis, while “S” denotes short axis. (A) Schematic of the tested directions, “L” and “S”. (B) Tensile modulus. (C) Yield stress. (D) Yield strain. All error bars denote standard deviation, significance was considered $*p \leq 0.05$, $n = 4$.

4.3.5. Towards complex scaffolds driving multiple tissue organisations

Having demonstrated the capacity of these MEW scaffolds to direct neotissue organisation, we next printed scaffolds containing two spatially distinct regions (here termed Region A and B) whose architectures were designed to mimic the inner and outer zone of native meniscus. Region A of the scaffold (0.2 x 3.2 mm MEW architecture) was designed to support the development of a highly aligned anisotropic collagenous tissue mimetic that of the outer zone of the meniscus. On the other hand, Region B (0.8 x 0.8 mm MEW architecture) was designed to support the development of a more isotropic tissue (Figure 4.7A,B). After culturing the constructs *in vitro* for 4 weeks, distinct tissue organisations were observed in each region of the scaffold, within the isotropic and anisotropic zones (Figure 4.7C). These ‘proof-of-concept’ results clearly demonstrate the potential of this convergence of MEW and inkjet bioprinting to engineer anisotropic tissues with spatially defined tissue organisation mimetic of native meniscal tissue.

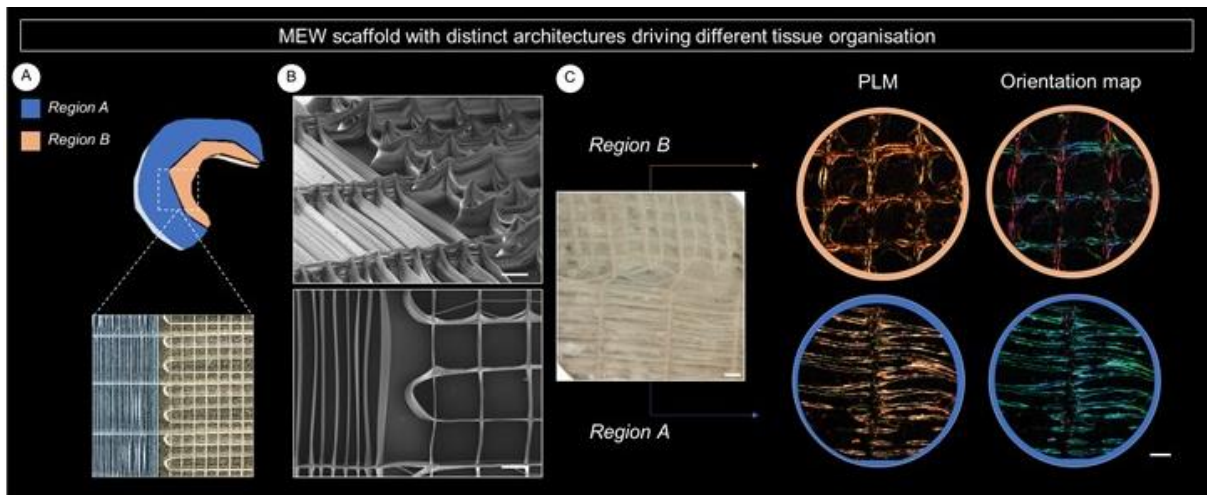


Figure 4.7. MEW scaffolds with regionally distinct architectures support spatially defined tissue organisation. (A) Schematic representation of the regions of the meniscus and the designed scaffold. (B) SEM images of the engineered scaffold. Scale bar is equal to 500 μm. (C) Bright field image (scale bar is equal to 800 μm) of the scaffold after 4 weeks of *in vitro* culture, and polarised light and colour map imaging of the collagen fibre distributions, scale bar is equal to 400 μm. Here, cyan/blue colours denote fibres oriented at 0 degrees, while purple/red denote fibres oriented at 90 degrees.

4.4. Discussion

Different biofabrication and bioprinting technologies have distinct benefits and limitations, motivating the development of convergent strategies that combine the benefits of different approaches in order to engineer complex biomimetic tissues [301, 322]. In the present work, we used inkjet bioprinting to deposit cell-laden bioinks into polymeric scaffolds produced by MEW and FDM, exploring how the architecture of the printed polymers influences neotissue organisation. We demonstrated that by changing the architecture of the microchambers in which the cells are physically confined, that it is possible to direct neo-tissue deposition, likely due to the physical boundaries imposed by the microchamber walls. We established that both additive manufacturing techniques were able to drive cartilage-like tissue formation, while MEW scaffolds further allowed for the biofabrication of neotissues with compressive properties similar to native meniscal tissue. Furthermore, the anisotropic mechanical properties of the engineered tissues could be modified by altering the aspect ratio of the microchambers. Finally, we demonstrated the feasibility of fabricating MEW scaffolds with regionally defined architectures capable of supporting the development of tissues with spatially distinct organisations. Taken together, these results demonstrate (1) how the architecture of MEW scaffolds can be tailored to direct collagen organisation and construct mechanical properties and (2) the potential of converging MEW and inkjet bioprinting for the engineering of anisotropic meniscal tissue.

By using a temporal bioink, based on oxidised alginate, we could provide temporal support for the cells after the jetting process. After crosslinking, the rapidly degrading hydrogel allowed the cells to self-organise following the microchamber architecture. The degradation properties of these hydrogel bioinks are characterised in detail in an earlier study [319], with complete degradation expected within 4 weeks. Furthermore, the use of oxidised alginate as a temporal bioink offers multiple opportunities to temporally and spatially localise cells and bioactive factors within a

bioprinted implant [135, 323]. Previously, we have shown how the integration of additive manufacturing approaches and inkjet bioprinting can promote the formation of structured arrays of cells [221, 244, 245]. By using polymeric frameworks, it was possible to guide the self-organisation of cells into biomimetic articular cartilage-like tissue [221, 244, 245]. In these previous studies, a permanent or temporary solid base was necessary for the spatial confinement of the printed cells within the polymeric microchambers, which was overcome in this study by using a sacrificial hydrogel to temporally support the cells, therefore, allowing us to generate implants with less polymer content which can take up to 4 years to degrade *in vivo* [188]. The use of such sacrificial hydrogel bioinks should also allow the bioprinting of thicker tissues, as it enables the controlled deposition of cells at specific heights into the supporting scaffold structures [319]. The use of MEW as a manufacturing technology yielded highly porous scaffolds with predefined geometries and polymeric fibres 15 μm in diameter. An important advantage of using MEW as a fabrication technique is therefore the increased porosity of the scaffold (as a result of the decreased filament size), which increases the space available for the formation of new tissue [198].

The architecture of both the FDM and MEW scaffolds was found to influence the alignment of the collagen network laid down by MSCs. It has previously been shown that the physical boundaries provided to cells by porous biomaterials guides subsequent tissue growth, following a so-called ‘curvature-driven growth mechanism’ [324, 325]. Specifically, it has been shown that tissue growth can be linked to distinct patterns of actin organisation and alignment, with greater alignment observed in narrower pores, suggesting a role for cell-generated forces in directing tissue growth in such porous scaffolds [324]. In the early stages of *in vitro* culture, we observed that MSCs align parallel to the microchamber walls, and at day 7 an elliptical growth pattern is observed, with cells predominately aligning parallel to the long axis of the microchambers. Others have reported similar patterns of cell alignment within MEW scaffolds [307]. In agreement with previous findings on porous structures produced via high-resolution

stereolithography [326], we also found that collagen alignment was dependent on the aspect ratio of the microchambers, with collagen preferentially aligning parallel to the long axis of the microchamber walls regardless of the fibre size. Hence, this multiple-tool biofabrication technique provides user-defined control of the collagen network architecture by providing well-controlled physical boundaries to the developing tissue. Of note, higher levels of alignment appear to occur around the edges of the microchambers. This effect can be explained by the fact that cells closer to the edge experience greater restrictions in their movement [327]. Conversely, cells located more towards the centre are subjected to fewer physical restrictions. The architecture of the MEW scaffolds also appeared to influence the rate of MSC proliferation and their biosynthetic activity. When comparing different architectures (e.g., 0.2x3.2 versus 0.8x0.8 MEW scaffolds), it may be that the higher aspect ratio supports a more proliferative, less biosynthetic cell phenotype (as evident by lower levels of matrix deposition). The smaller spacing between the microchamber walls in the 0.2x3.2 mm group may help cellular bridging across the fibre walls, allowing a higher rate of cell proliferation [201]. Another factor that could explain the observed differences between FDM and MEW is that the number of jetted cells per microchamber was adjusted to maintain a constant seeding number per scaffold. Consequently, the number of cells in each microchamber varied across the groups. However, to gain a deeper understanding of the biological mechanism and significance, further validation involving multiple donors is necessary. Additionally, future studies would benefit from incorporating additional microscopy analysis to enhance the understanding of the interactions between cells and scaffolds.

As expected, the compressive stiffness of the bioprinted constructs generated using FDM scaffolds was orders of magnitude stiffer than the native meniscus, while those generated using MEW were comparable to the native tissue [321]. While it is possible to reduce the bulk compressive properties of FDM scaffolds by altering the laydown pattern of the printed fibres [191], this changes the aspect ratio of the

microchamber walls (i.e. the geometry of the scaffold pores) and hence could limit the ability to use the same printed structures to guide tissue growth and organisation in specific directions. The MEW scaffolds also possess a lower porosity and consist of PCL fibres with higher aspect ratios more mimetic of the collagen fibres in biological tissue, making them more attractive for soft tissue engineering applications. For these reasons, we decided to progress with the use of the MEW scaffolds over the FDM scaffolds in our attempts to bioprint anisotropic constructs with biomimetic mechanical properties in both tension and compression. The meniscus possesses an anisotropic tensile behaviour due to the circumferentially oriented collagen fibres. By varying the aspect ratio of the microchambers, it was possible to bioprint constructs with a similar anisotropic behaviour, being stiffer parallel to the long (circumferential) axis compared to and short (radial) axis. Although the neomatrix deposited within these MEW scaffolds enhanced the tensile strength of the resulting constructs, they were still noticeably less than the reported average values for the human meniscus, which ranges between 60 and 200 MPa in the circumferential direction [310]. Although it is not trivial to achieve native values in highly porous scaffolds fabricated with small fibre diameter biomaterials, further improvements in tissue mechanical properties could possibly be achieved by the application of external factors such as chondroitinase-ABC or lysyl oxidase, which has previously been shown to increase the tensile properties of engineered cartilage tissues [170, 177, 178, 328]. The overall porosity of the MEW scaffold could also be slightly reduced if further increases in tensile properties are deemed necessary.

In this work, MSCs were used as a cell source due to their ease of availability and ability to produce a fibrocartilage-like tissue *in vitro*. Over 8 weeks in culture, MSCs secreted a tissue rich in sGAGs and type I and type II collagen. Bone marrow-derived MSCs possess certain advantages and disadvantages over meniscus-derived cells for meniscus tissue engineering. MSCs have been shown to generate a more ECM-rich tissue with superior mechanical properties compared to meniscus-derived cells, however their tendency to generate a hypertrophic cartilaginous tissue remains a major concern [329].

It has also been shown that while MSCs support high levels of matrix production, they lack the fibre organisation capabilities of primary fibrochondrocytes [172, 173]. Enzymatic treatments could potentially be used to improve collagen fibre formation in tissues generated using MSCs [173].

The different microchamber architectures promoted distinct cartilage phenotypes, with decreased collagen type X expression in microchambers with larger aspect ratios. Type X collagen is a common marker of hypertrophy and progression along an endochondral pathway. Moreover, larger aspect ratios resulted in increased type I and reduced type II collagen deposition compared to the other architectures, indicating higher aspect ratios promote a more fibrochondrogenic phenotype. Others have also shown that topographical cues can induce distinct MSC phenotypes in the presence of TGF- β 3 [238], with aligned electrospun scaffolds supporting a hyaline cartilage phenotype, while randomly aligned fibrous scaffolds promoted progression along the endochondral pathway. Together these observations indicate that scaffold architecture can be used to control both the organisation and phenotype of the engineered cartilage tissue. Further work is required to identify specific combinations of scaffold architectures and biochemical stimulation regimes to support the distinct phenotypes observed in the inner and outer zones of the meniscus. Alternatively, it would be possible to deposit phenotypically distinct meniscus progenitor cells into different regions of an appropriately designed MEW scaffold to better engineer zonally defined meniscal grafts. A population of progenitor cells has been identified in the meniscus tissue, which may be particularly suited to cell-based approaches to meniscus regeneration [114]. Ideally these would be deposited into MEW scaffolds of relevant anatomical size [330, 331], potentially enabling the bioprinting of human scale meniscal grafts with biomimetic structure and composition. Finally, other researchers have fabricated meniscus-shaped PCL scaffolds containing hydrogels which encapsulated cells and poly(lactic-co-glycolic acid) (PLGA) microparticles carrying TGF- β 3 or (CTGF) resulting in anisotropic phenotypes in different regions on the engineered constructs [164, 293]. This combination

of scaffold microarchitectural and biochemical signalling could result in an interesting approach for the fabrication of meniscal grafts that mimic the morphology, mechanics and microenvironment.

4.5. Conclusion

We have developed a convergent bioprinting strategy that enables the engineering of soft tissues with user-defined collagen network architectures and anisotropic mechanical properties. By increasing the aspect ratio of the printed scaffold microchambers, it was possible to preferentially direct collagen deposition parallel to the long axis of these guiding structures. The resulting tissues mimicked key aspects of the mechanical behaviour of native meniscus tissue. We also demonstrated the potential of MEW to generate spatially inhomogeneous scaffolds capable of controlling the alignment of the tissue in multiple directions. This multiple-tool biofabrication strategy constitutes, therefore, the basis of a promising approach for the development of biomimetic meniscus implants.

Chapter 5: Chondroitinase ABC treatment improves the organisation and mechanics of 3D bioprinted meniscal tissue

5.1. Introduction

The meniscus is a crescent-shaped fibrocartilage tissue that is integral to the functionality of the knee joint, acting to maintain stability and distributing loads across the joint surface [26, 48, 63]. The mechanical function of the meniscus is tightly linked to the unique architecture of the collagen fibres within the tissue, which are preferentially oriented in the circumferential direction, enabling the tissue to bear high levels of load [4]. Injuries to the meniscus disrupt the normal functioning of the knee joint and can eventually lead to the development of osteoarthritis [9]. Current clinical treatments for damaged menisci, such as partial or total meniscectomy, only provide short-term relief [49]. This has led to increased interest in the development of biomaterial implants to provide structural support and serve as a template for new tissue formation [35, 37]. However, none of the clinically available biomaterial substitutes closely recapitulate the intricate organisation of the native meniscus that is key to its correct functionality. In this context, 3D bioprinting is emerging as a biofabrication technique with the ability to produce regenerative constructs that mimic the composition and key architectural features of living tissues [223].

Different 3D bioprinting strategies have already been employed to produce biomimetic constructs for meniscus tissue engineering [51, 52]. A common approach has been to use synthetic polymers to fabricate porous scaffolds with bulk mechanical properties comparable to the native tissue [2, 42, 332, 333]. Such scaffolds can be further functionalised with factors supportive of tissue regeneration; for example, the inclusion of growth factor-containing microspheres at specific locations within polycaprolactone

(PCL)-based scaffolds has been shown to support the development of zone-specific cellular phenotypes mimetic of the native meniscus [3]. It has also been demonstrated that the application of both biochemical and biomechanical stimuli to cell-seeded PCL scaffolds can synergistically enhance meniscus zonal organisation *in vitro* and *in vivo* [8]. Cellular constructs have been fabricated by sequentially printing cell-laden bioinks within supporting PCL scaffolds [164, 334]. The majority of such 3D printed grafts aim to replicate certain aspects of the native tissue anisotropy through the circumferential patterning of polymeric materials like PCL. Although this enables the production of scaffolds with promising bulk mechanical properties, other features of the tissue (e.g. tension-compression non-linearity) are difficult to replicate, and the key challenge of engineering grafts with a collagen architecture mimetic of the native meniscus has yet to be adequately addressed [178].

The structural guidance of cells by physical cues is a powerful approach to direct tissue formation and maturation [335, 336]. Diverse strategies, from the use of microgrooves to the electrospinning of aligned fibrous polymers, have been explored as physical guiding templates to control cellular orientation and/or collagen organisation [161, 238, 324, 337]. However, such approaches are generally not suitable for the biofabrication of large anatomically defined implants, which is particularly important for functional meniscus regeneration, thereby motivating the use of emerging 3D bioprinting strategies to address this challenge [338]. We have previously reported a convergent bioprinting strategy that enables the engineering of soft tissues with user-defined collagen network architectures and anisotropic mechanical properties [221, 244, 245]. Using melt electrowriting (MEW) to produce scaffolds with precise, predefined architectures, we were able to alter the physical boundaries imposed upon cells and their secreted extracellular matrix. In MEW scaffolds with highly elongated pore architectures, the deposited ECM preferentially aligned parallel to the long axis of the printed pores, enabling the bioprinting of tissues with collagen organisation somewhat mimetic of the native meniscus. Although the compressive properties of the resulting tissues were within

the range of native values (90-160 kPa) [64], the tensile properties, which are integral to tissue functionality, were still orders of magnitude below native values. It has previously been shown that the application of enzymes like chondroitinase ABC (cABC), which temporally remove sulphated glycosaminoglycans (sGAGs) from the developing tissue, can enhance collagen fibre maturation and hence increase the mechanical properties of engineered cartilage and meniscus [170, 174, 175, 178, 339-341]. In the context of articular cartilage tissue engineering, it has also been shown that such treatments can support the formation of a more zonally organised collagen architecture [173]. Therefore, the goal of this study was to determine the impact of such enzymatic treatment on the composition, structural organisation and biomechanics of meniscal tissues that are engineered by inkjetting mesenchymal stem/stromal cells (MSCs) into MEW scaffolds with aligned pore architectures that support the development of structurally anisotropic fibrocartilaginous grafts.

5.2. Material and methods

Melt Electrowriting (MEW): 1 mm in height MEW scaffolds with a fibre diameter of 15 μm were fabricated on a custom MEW printer built in house [24, 25]. Briefly, PCL (50,000 MW, CAPA 6500D, Perstorp) was melted at a temperature of 80 °C and 85 °C at the barrel and around the nozzle (21G) respectively. PCL was extruded at a pressure of 0.6 Bar with an initial voltage of 6 kV at a distance of 6 mm from the grounded aluminium collector plate, and a translational speed of 6 mm/s. Fibres were subsequently deposited with the predesigned architecture.

Isolation and Expansion of Mesenchymal Stem/Stromal Cells: Bone marrow-derived MSCs (bMSCs) were harvested from the femur of a 4-month old pig supplied by a local abattoir, as previously described [342]. Briefly, the bone marrow was collected under sterile conditions from the femoral shaft, and diluted with expansion medium (XPAN), consisting of hgDMEM supplemented with 10% v/v FBS, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 2.5 $\mu\text{g/mL}$ amphotericin B (all Gibco, Biosciences). After

obtaining a homogenous suspension using a 16G needle and filtering through a 40- μ m nylon mesh, cells were counted using trypan blue containing 3% acetic acid before plating into T175 flasks for expansion. All expansion was performed at 5% pO₂, and using XPAN medium supplemented with 5 ng/ml FGF2 (Peprotech), seeding at a density of 875.000 cells per T175 flask. Chondrogenic, osteogenic and adipogenic differentiation assays were used to assess the tripotentiality of isolated MSCs. Cells were used at the end of passage 3 for all experiments.

Oxidised Alginate Preparation (OA): The alginate oxidation was performed as previously described [343]. Briefly, 1 g of alginate (MVG, Pronova Biopolymers) was dissolved in deionised water overnight at RT and mixed with sodium periodate (Honeywell) to achieve a theoretical alginate oxidation of 4% under stirring in the dark at room temperature for 24 h. The oxidised alginate was then purified by dialysis against deionised water for 3 days (MWCO 3500 Dalton; Fischer), sterile filtered through a 0.22 μ m filter, and lyophilised to obtain the final product.

Inkjet bioprinting and chondrogenic culture: The inkjet head of the 3D Discovery multi-head printing system (RegenHU) was used to deposit a cell-alginate bioink (3x10⁷ cells/mL in 3% (w/v) oxidised alginate). Single droplets were deposited into each well to yield 17x10³ cells/microchamber (using a valve opening time of 600 μ s and a pressure of 0.1 MPa). Post-printing, a 45 mM CaCl₂ solution in hgDMEM was added to crosslink the alginate for 5 min at room temperature. The crosslinking solution was then removed and replaced by XPAN medium. After a period of 24 h, all constructs were moved to chondrogenic differentiation medium (CDM), which consisted of hgDMEM supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin (both Gibco), 100 μ g/mL sodium pyruvate, 40 μ g/mL L-proline, 50 μ g/ml L-ascorbic acid-2-phosphate, 4.7 μ g/ml linoleic acid, 1.5 mg/ml bovine serum albumin, 1 $\times$ Insulin–Transferrin–Selenium (ITS), 100 nM dexamethasone, 2.5 μ g/ml Amphotericin B (all from Sigma) and 10 ng/ml

of human transforming growth factor- β 3 (TGF- β) (Peprotech). Cells were cultured at 5% pO₂ for 5 weeks with medium changes performed every two days.

Chondroitinase-ABC Treatment: On day 14 of the chondrogenic culture, constructs were treated with an enzymatic solution consisting of 2 U/mL cABC (Sigma-Aldrich), 0.05 M acetate (Trizma Base, Sigma-Aldrich) activator in hgDMEM for 4 hours at 5% pO₂. After the enzymatic treatment, the engineered tissues were washed with hgDMEM before the addition of CDM and the continuation of the culture period. This enzymatic treatment protocol is based on previous work in the literature, and has proven beneficial effects previously in our lab [173, 174, 340, 341].

Live/Dead Imaging: Cell viability was assessed using the live/dead assay. Briefly, constructs were washed in PBS followed by incubation for 1 h in PBS containing 2 μ M calcein acetoxymethyl (calcein AM) and 4 μ M ethidium homodimer-1 (EthD-1) (both from Bioscience) for 1 h. Samples were then washed in PBS before imaging with a Leica SP8 scanning confocal microscope excited at 494 nm and 528 nm, and read at 517 nm and 617 nm.

Biochemical Analysis: The engineered constructs were biochemically analysed after 5 weeks of *in vitro* culture. After serial washes with PBS, each construct was digested with papain (3.88 U/mL) in ultra-pure water containing 0.1 M sodium acetate, 5 mM L-cysteine–hydrochloride hydrate, 5 mM methylenediaminetetraacetic acid (EDTA) (all from Sigma). Samples were incubated in papain solution pH 6.5 at 60 °C under rotation for 18 h. Immediately after digestion, the DNA content was quantified using the Hoechst 33258 dye assay with calf thymus DNA as standard (Merck) reading at 360 nm excitation and 460 nm emission. The amount of sulphated glycosaminoglycan (sGAG) was quantified using the 1,9-dimethylmethylene blue (DMMB) dye-binding assay, with a chondroitin sulphate solution as standard (Blyscan) 28. The 530/590 absorbance ratio was used to generate the standard curve and determine the sGAG concentration in the digested samples. Total collagen content was determined by measuring the hydroxyproline content

using the dimethylaminobenzaldehyde and chloramine T assay, and a hydroxyproline/collagen ratio of 1:7.69 [273]. Briefly, samples were mixed with 100 μ L of 38% HCl, and incubated at 100 °C for 18 h. After cooling, samples were centrifuge at 5000 g for 5 min, and let to dry at 50 °C for 48 h. Dried samples were then dissolved in 200 μ L ultra-pure water. 2.82 % (w/v) chloramine T solution was added and incubated for 20 min at room temperature in the dark, before the addition of a 50 % (w/v) 4-(Dimethylamino) benzaldehyde solution (both Sigma) at 60 °C for 20 min. Hydroxyproline levels were estimated from the standard curve at a wavelength of 570 nm.

Histological Analysis: Constructs were fixed in 4% (w/v) paraformaldehyde, dehydrated in a graded series of ethanol and xylene baths, embedded in paraffin wax, sectioned at 8 μ m using a microtome (Leica Microsystems), and affixed to microscope slides. The sections were stained with haematoxylin and eosin (H&E), alcian blue, picrosirius red and alizarin red. For immunohistochemistry antigen retrieval was carried out by an initial treatment with pronase (3.5 U/ml; Merck) at 37 °C for 25 min, followed by hyaluronidase (4000 units/ml; Sigma-Aldrich) at 37 °C for 25 min for collagen type I and type II. For collagen type X, the antigen retrieval method consisted of an initial treatment with pronase (35 U/mL; Merck) at 37 °C for 5 min, followed by chondroitinase ABC (0.25 U/mL; Sigma-Aldrich) at 37 °C for 45 min. Non-specific sites were blocked using a 10% goat serum and 1% BSA blocking buffer for 1 h at room temperature. Collagen type I (1:400; ab138492; Abcam), type II (1:400; sc52658; Santa Cruz), and type X (1:300; ab49945; Abcam) primary antibodies were incubated overnight at 4 °C, followed by 20 min treatment using a solution of 3% hydrogen peroxide (Sigma-Aldrich). The secondary antibodies for collagen type I (1:250; ab6720; Abcam), type II (1:300; B7151; Sigma-Aldrich) and type X (1:500, ab97228; Abcam) were incubated for 1 h at RT. Samples were then incubated for 45 min with VECTASTAIN Elite ABC before treating them with ImmPACT DAB EqV (both from Vector Labs) at RT. Slides were then imaged using an Aperio ScanScope slide scanner, while sections stained with picrosirius red were imaged

using polarised light microscopy. Analysis on the fibre orientation and coherency was carried out using the OrientationJ plugin in ImageJ software [344].

Scanning Electron Microscopy: For SEM imaging, samples were fixed in 3% glutaraldehyde overnight, and dehydrated in graded ethanol series before immersing the samples into hexamethyldisilazane (HMDS). After drying, samples were mounted on SEM pin stubs with carbon adhesive discs and coated with gold/palladium for 60 s at a current of 40 mA using a Cressington 208HR sputter coater. Imaging was carried out in a Zeiss ULTRA plus SEM.

Scanning Helium Ion Microscopy: For high resolution visualisation of the collagen network, samples were image with a scanning helium ion microscope (Zeiss ORION Nanofab). At the end of the culture period, samples were treated with 0.6 U/mL cABC for 24 h at 37 °C, followed by a treatment with 20.4 U/mL hyaluronidase for another 24 hours at 37 °C [312]. After the enzymatic treatment, samples were washed in PBS, and rinsed with diH₂O. Finally, samples were dehydrated in a gradient of series of ethanol and dried at a critical point. Samples were then mounted and coated as previously described and imaged using a Zeiss ORION Nanofab.

Mechanical Testing: To investigate the mechanical properties of the engineered tissues the different groups were tested using a single column Zwick (Zwick, Rowell) with a 10 N load cell at room temperature. Unconfined compression tests were carried out in a PBS bath as previously described [274]. The Young's modulus was defined as the slope of the linear phase of the resulting stress-strain curve during the ramp phase of the compression to 10% strain. The equilibrium modulus was determined as the force in equilibrium divided by the sample's cross-sectional area divided by the applied strain, while the dynamic modulus was measured as the average force amplitude over the five cycles divided by the sample's cross-sectional area divided by the applied strain amplitude [191]. Uniaxial tensile tests were performed into rectangular sections as previously described

[195]. The elastic modulus was calculated from the stress-strain curves in the linear region.

Statistical Analysis: Statistical analysis was performed using GraphPad Prism software. Statistical differences were determined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or Student's t-test, where appropriate. Results are displayed as mean $\pm$ standard deviation. Significance was accepted at a level of $p \leq 0.05$. Sample size (n) is indicated within the corresponding figure legends.

5.3. Results

5.3.1. Integrating MEW and the inkjet bioprinting of cell-laden bioinks

A previously optimised biofabrication process was used to control the deposition of MSCs into MEW scaffolds [345]. Briefly, a MEW process was first used to produce fibrous PCL scaffolds with a pore size of 0.4 mm by 1.6 mm and a scaffold thickness of 1 mm (Figure 5.1A). To this end, polycaprolactone (PCL) was melted at 80 °C and deposited using a starting electric field of 6 kV and a pressure of 0.6 Bar, resulting in a scaffold consisting of an array of microchambers of defined size. Scanning electron microscopy (SEM) of the scaffolds revealed the precise stacking of PCL fibres, with an average fibre diameter of 15 μm (Figure 5.1B). MSCs within a supporting oxidised alginate bioink were next jetted into each scaffold pore (here termed 'microchambers') of these guiding MEW scaffolds [244]. Approximately 0.56 μL of this ink was deposited into each microchamber, with each droplet containing 17,000 cells. Live/dead imaging indicated that the cells remained viable 7 days after the biofabrication process (Figure 5.1C). SEM analysis revealed that the cells were able to interact with and deform the PCL microfibres (Figure 5.1D).

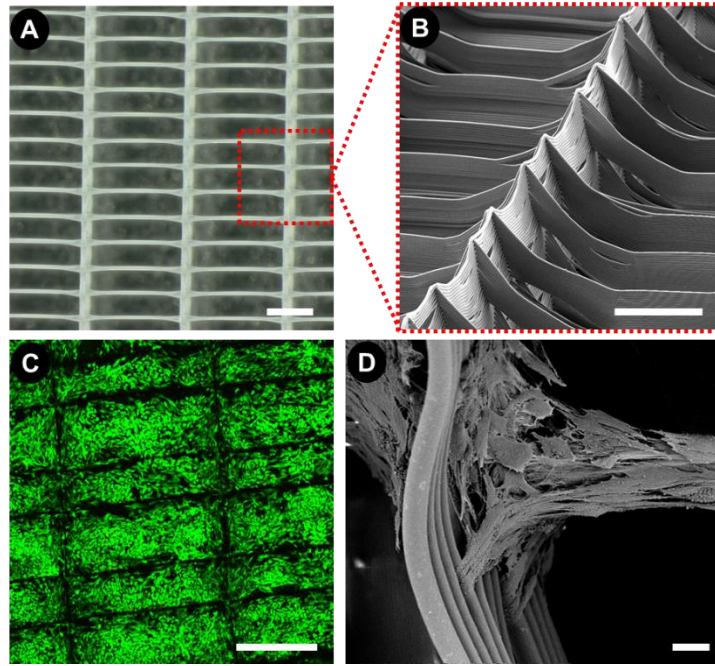


Figure 5.1. Development of the guiding microchamber system and inkjet bioprinting. (a,b) Stereomicroscope and SEM images of the MEW scaffold. Scale bars are equal to 800 μm . (c) Live/dead images (z-stacks) at day 7 after the inkjet step. Scale bar is equal to 800 μm . (d) SEM images of the cells interacting with the MEW scaffold. Scale bar is equal to 20 μm .

5.3.2. Enzymatic treatment supports the development of a more collagenous rich engineered tissue

We have previously demonstrated that appropriately designed MEW scaffolds can support the development of structurally organised fibrocartilage when seeded with MSCs and maintained in chondrogenic culture conditions [345]. In an attempt to support collagen fibre maturation, here we investigated the effect of temporal enzymatic treatment on the functional development of such engineered fibrocartilaginous tissues. To this end, we exposed the engineered tissue to chondroitinase ABC (cABC) solution for 4 hours on day 14 of chondrogenic culture. As expected, biochemical analysis after 5 weeks of culture indicated that cABC treatment significantly reduced total sGAG levels compared to non-treated controls (Figure 5.2B). cABC treatment had no effect on the total levels of DNA or collagen (Figure 5.2A, 5.2C). Enzymatic treatment was also found to alter the

ratio of sGAG to collagen within the engineered tissue, supporting the development of a more collagen rich matrix typical of the native meniscus (Figure 5.2D) [63].

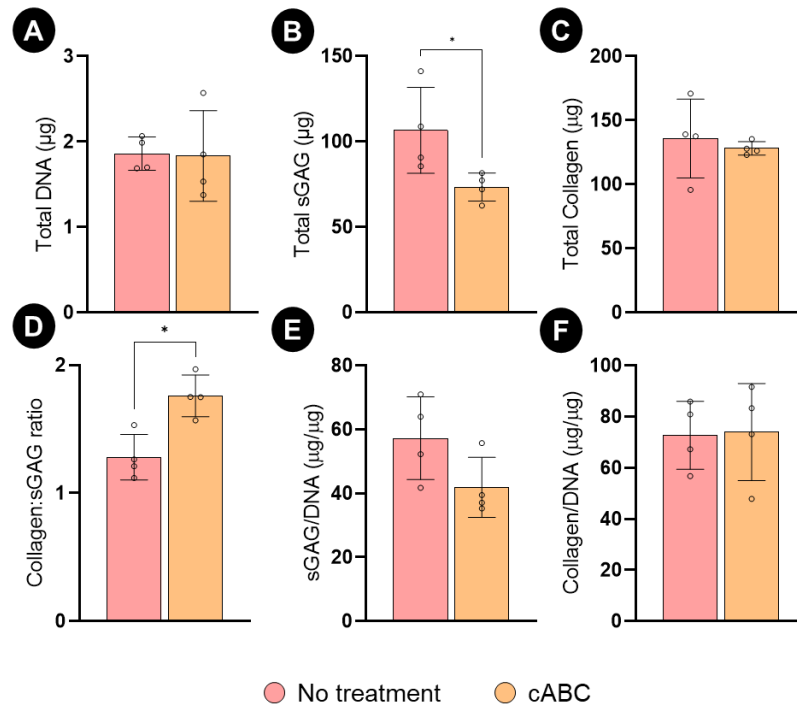


Figure 5.2. Biochemical properties of the engineered cartilage tissue following 5 weeks of *in vitro* culture. Quantification of (a) DNA, (b) sGAG, and (c) collagen. (d) Ratio collagen/sGAG. (e) sGAG and (f) collagen content normalised to amount of DNA per construct. All error bars denote standard deviation, significance was considered $p \leq 0.05$, $n = 4$.

Having identified changes in ECM composition following the enzymatic treatment, we further investigated how cABC treatment influences tissue phenotype using histology and immunohistochemistry. As expected alcian blue staining, which stains for sGAGs, was lighter in the treatment group (Figure 5.3). No evidence of calcium deposition (a marker of tissue hypertrophy) was observed in either group. To further probe a fibrocartilage phenotype, immunohistochemical staining for collagen types I, II and X were undertaken. All engineered cartilage stained intensely for collagen type I and II. Collagen type X was expressed at lower levels, with staining observed predominantly at the edges of the microchambers. This suggests that cABC treatment does not have a major impact on the tissue phenotype.

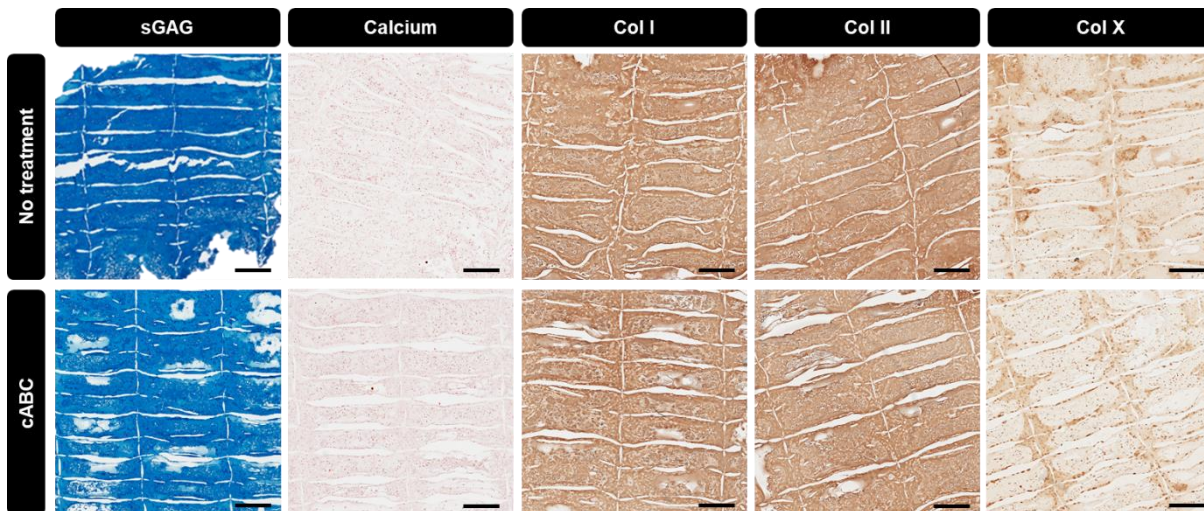


Figure 5.3. Histological analysis of the engineered cartilage tissue following 5 weeks of *in vitro* culture. Stained for alcian blue (sGAG), alizarin red (Calcium), collagen type I, II and X. Scale bar is equal to 800 μm .

5.3.3. Enzymatic treatment influences collagen network maturation

Polarised-light microscopy (PLM) was next used to investigate if enzymatic treatment influences the spatial organisation of the engineered tissue. Although the intensity of picrosirius red (PR) staining for collagen deposition appeared unaffected by cABC treatment, it did result in a clear change of intensity in the colour of the collagen fibres when viewed under polarised light (Figure 5.4A). This higher intensity is typically indicative of thickening of the collagen fibres, suggesting that cABC treatment was supportive of increased collagen fibre maturity [346]. cABC treatment did not appear to influence collagen network alignment, with collagen fibres in the treated and untreated groups displaying clear preferential directionality parallel to the long axis of the MEW microchambers (Figure 5.4B). Fibre coherency, which indicates the variance of the collagen fibre distribution, was not statistically different between groups (Figure 5.4C). Collectively, these results suggest that cABC does not alter the fibre distribution, but that it does affect fibre maturation.

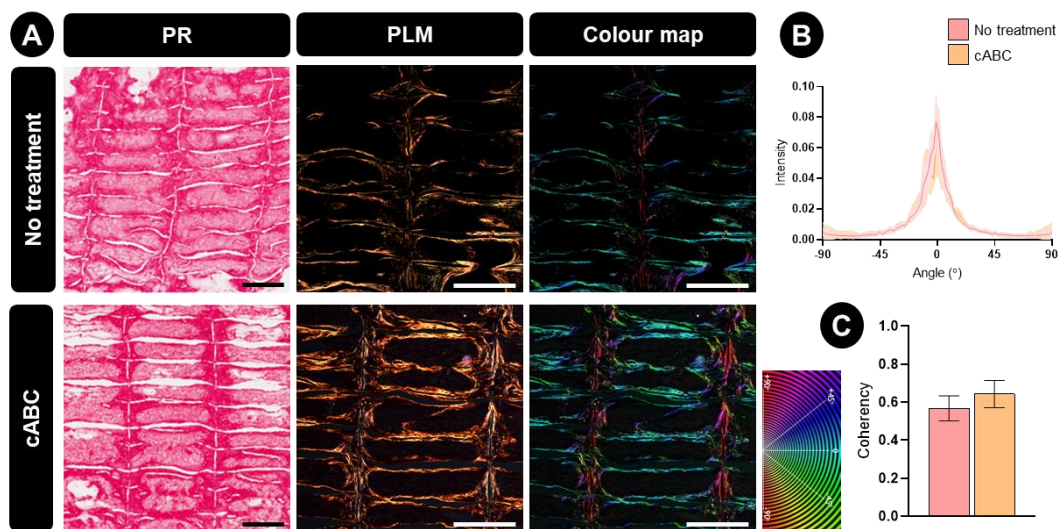


Figure 5.4. Collagen organisation within the engineered tissues following 5 weeks of *in vitro* culture. (A) Picrosirius red staining, polarised light and colour map imaging of collagen fibre distributions. Scale bar is equal to 800 μm . (B) Collagen fibre directionality. (C) Fibre coherency quantification, where a value of 1 indicates fibres are aligned in the same direction, while a value of 0 indicates dispersion of fibres in all directions. All error bars denote standard deviation.

To further evaluate the influence of cABC treatment on collagen network development, the engineered tissues were analysed by SEM (Figure 5.5A). The resulting images were quantified to determine collagen fibre diameter. Collagen fibre diameter in the non-treated groups was found to be 128 ± 52.23 nm, whereas with the cABC treatment the average fibre size increased by $\sim 78\%$ to 229 ± 69.58 nm (Figure 5.5B). These results indicate that a one-time enzymatic treatment has a potent effect on collagen fibre maturation.

5.3.4. Enzymatic treatment increases the mechanical properties of bioprinted fibrocartilage tissue compared to empty scaffolds.

Biomechanical testing was carried out to understand the influence of cABC treatment on the functional development of the engineered tissue. No significant influence of cABC treatment was observed during unconfined compression testing, although there was a

trend towards cABC treatment increasing the compressive ramp modulus of the tissue (Figure 5.6A). On the other hand, there was a trend towards a lower equilibrium modulus following cABC treatment, which is to be expected given the drop in sGAG content in this group (Figure 5.6B, C). Finally, only cABC treated tissues were found to have a tensile modulus that was significantly higher than that of an empty MEW scaffold after 5 weeks of culture (Figure 5.6D).

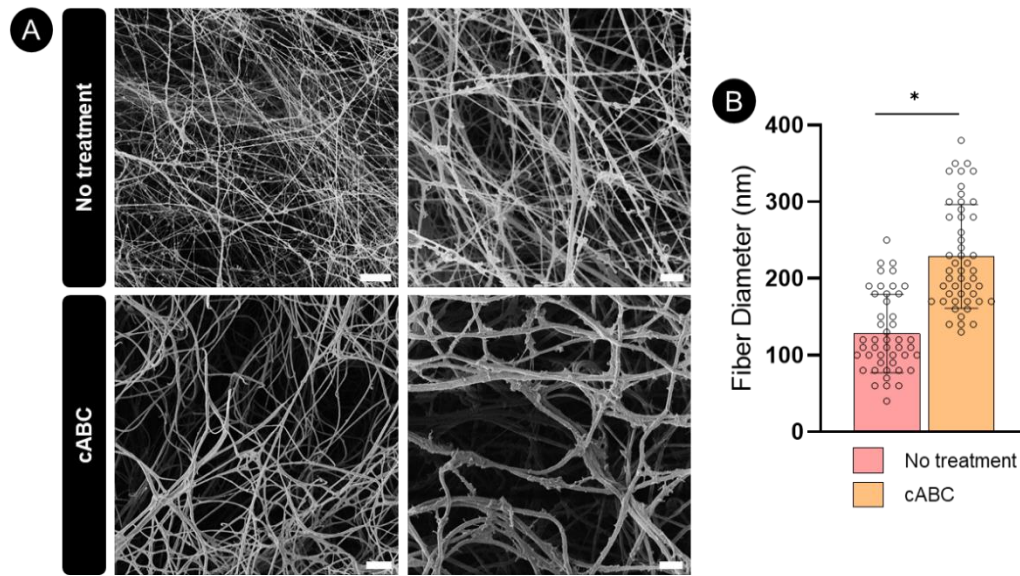


Figure 5.5. SEM analysis of the collagen organisation within the engineered tissues. (A) Representative SEM images. Scale bar is equal to 5 μm on the left, and 1 μm on the images on the right. (B) Quantification of the collagen fibre diameter. All error bars denote standard deviation, significance was considered $p \leq 0.05$.

5.4. Discussion

This study focused on investigating the effects of cABC treatment on collagen network development in bioprinted fibrocartilaginous tissue. We had previously shown that by modifying the architecture of MEW scaffolds, specifically the aspect ratio of its pores (or microchambers), that it was possible to control the directionality of collagen fibres laid down by cells jetted into such constructs [345]. However, the tensile properties of such engineered tissues were at least an order of magnitude below native values. Here we hypothesised that a one-time enzymatic treatment would positively impact the maturation

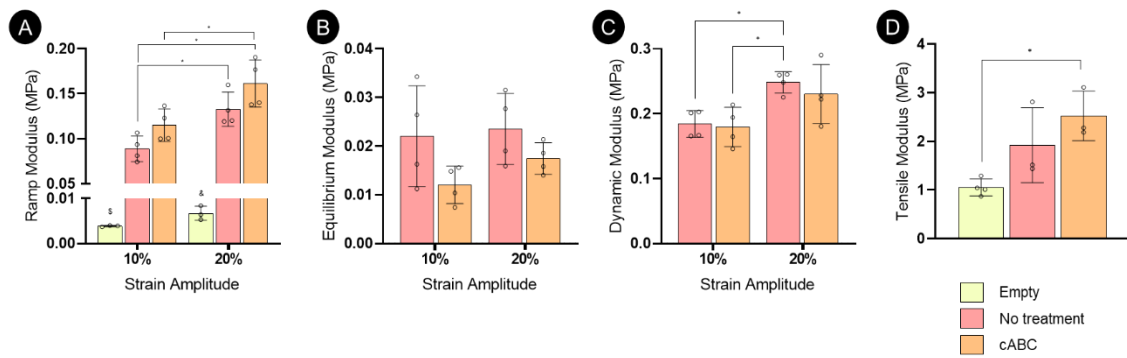


Figure 5.6. Biomechanical properties of the engineered tissues following 5 weeks of *in vitro* culture. (A) Ramp modulus. (B) Equilibrium modulus. (C) Dynamic modulus. (D) Tensile modulus. All error bars denote standard deviation, significance was considered $p \leq 0.05$, $n = 4$ for the compression tests and $n = 3$ for the tensile samples.

of the collagen network and hence improve the biomechanical properties of the engineered graft. We found that the temporal depletion of sGAGs within the engineered tissue following cABC treatment was associated with an increase in collagen fibre diameter without any detrimental effect on the tissue phenotype or collagen fibre directionality. Furthermore, this increase in collagen fibre thickness correlated with superior biomechanical properties compared to empty scaffolds.

MEW allows for the fabrication of well-defined polymeric scaffolds with specific geometries [193, 313]. Due to residual charges around the deposited fibres, it can be challenging to produce large scale scaffolds using this additive manufacturing approach. However, iteratively increasing the electric field strength during the manufacturing process can overcome such challenges [313, 347, 348]. In this work, we fabricated 1mm thick scaffolds with a fibre thickness and a pore architecture that has previously been shown to direct collagen fibre alignment parallel to the long axis of the scaffold pore walls [345]. After jetting MSCs into the MEW scaffolds, we observed a homogenous distribution of cells within the pores of the scaffold. Our work indicates that this convergence of biofabrication strategies (MEW and inkjetting) is a potent strategy for the spatial patterning of cells within large MEW scaffolds with user-defined architectures, potentially enabling the engineering of spatially complex tissues.

Although this multiple-tool biofabrication strategy enables the engineering of spatially organised tissues at a millimetre scale, it is still challenging to produce constructs with collagen content and mechanical properties comparable to native tissues. Here, we demonstrate that the use of cABC as a remodelling enzyme reduces the total sGAG content within the engineered tissue, thereby modulating the ratio of collagen to sGAG without compromising cell viability. Since the native meniscus is a predominantly collagen rich tissue, such treatments support the engineering of tissues that better recapitulate the biochemical content of the native meniscus [23]. In line with previous work, we observed that the enzymatic treatment did not negatively impact subsequent ECM synthesis [170, 328, 339]. Furthermore, enzymatic treatment had no effect on the resulting tissue phenotype, specifically the types of collagen deposited within the MEW scaffolds. No evidence of calcium deposition, a marker of hypertrophy and progression along an endochondral pathway, was observed in either group. Therefore, the use of cABC during chondrogenic differentiation represents a potent approach to modulate the biochemical composition of the engineered tissues without any detrimental effect on cell phenotype.

Temporal enzymatic treatment was associated with the development of larger diameter collagen fibres within the engineered tissue, as evident by polarised light microscopy and SEM. The presence of proteoglycans is known to impact collagen fibril morphology and formation kinetics by hindering lateral growth during fibril formation, leading to suggestions that their removal by cABC treatment can enhance collagen network development in engineered tissues [170, 171, 349]. Furthermore, it has been shown that increasing sGAG content is inversely correlated with decreasing collagen fibril size, indicating that sGAGs are important regulators of collagen network formation [172]. Importantly for the engineering of structurally organised fibrocartilage tissue, such changes in collagen fibril diameter did not have any detrimental effect on collagen alignment within the MEW scaffolds. This meant that cABC treatment not only enhanced collagen network maturity, but also increased the tensile properties of the graft. In spite

of these improvements, the tensile properties of the bioprinted meniscal grafts were still substantially lower than that of the native tissue. In the healthy meniscus, the collagen fibrils are organised together in bundles approximately 20-200 μm wide [4]. Therefore, while temporal enzymatic treatment represents a promising strategy to promote collagen network maturation, additional biochemical and biophysical cues are likely required to promote further tissue development *in vitro*.

5.5. Conclusion

In conclusion, this work describes a biofabrication strategy to direct collagen fibre alignment and maturation in bioprinted fibrocartilage tissue, where temporal depletion of sGAGs through enzymatic treatment with cABC facilitates the assembly of larger diameter collagen fibres. Chondrogenically primed MSCs produce high levels of sGAG in culture, which appears to impact collagen fibre formation and the functional development of the engineered tissue. The application of cABC to sGAGs in the early stages of tissue development supports an increase in collagen fibre size and graft mechanical properties without any detrimental effect on cell viability or tissue phenotype. These findings support the inclusion of enzymatic treatments when bioprinting fibrocartilaginous tissues as a means of generating more functional and biomimetic grafts.

Chapter 6: Bioprinting of scaled-up meniscal grafts by spatially patterning phenotypically distinct meniscus progenitor cells within melt electrowritten scaffolds

6.1. Introduction

The knee joint menisci are wedged-shaped fibrocartilaginous tissues located between the femoral condyle and the tibia plateau [63, 350]. They play a key role in maintaining stability and in the distribution of loads across the joint, thus protecting the underlying articular cartilage during normal daily activities [4]. The meniscus is often classified into two different regions, an inner avascular region, and an outer vascularised region. While the outer region is predominantly composed of circumferentially aligned type I collagen fibres and populated with fusiform-shaped fibroblast-like cells, the inner avascular region is compositionally more similar to articular cartilage and contains a higher proportion of type II collagen [38, 57]. This unique structure and composition provide the tissue with mechanical properties ideally suited to its function. In spite of this, injuries to the meniscus are common, with an annual incidence rate of 66 per 100,000 people in the United States [67, 351]. Injuries to the meniscus alter joint homeostasis and significantly increase the risk of developing osteoarthritis later in life [19, 352]. For that reason there is increased interest in surgical meniscus repair, with factors such as the type, geometry and location of the injury impacting clinical outcomes [18]. Furthermore, in many cases surgical repair is not feasible, motivating the need for innovative strategies to direct meniscal regeneration.

3D bioprinting is emerging as a particularly promising strategy in the field of tissue engineering and regenerative medicine as it allows for the controlled deposition

and assembly of biomaterials, bioactive factors and cells, resulting in the development of constructs that mimic key aspects of the structure and composition of native tissues [52, 126, 258, 353, 354]. Such approaches have been applied to the engineering of meniscal constructs, with the 3D printing of synthetic polymers such as polycaprolactone (PCL) commonly used to fabricate implants with bulk mechanical properties comparable to the native tissue. Furthermore, spatial functionalisation of such scaffolds with bioactive factors has been shown to support the formation of meniscus-like tissues both *in vitro* and *in vivo* [3, 42, 190]. Bioinks supportive of the different regions of the meniscus have also been developed; for example, meniscus extracellular matrix (ECM) based bioinks have been shown to support the development of meniscus-specific phenotypes [2, 146, 355, 356]. The limited self-healing capacity of the meniscus also suggests that the incorporation of a cellular component will be required for a successful regenerative implant [146, 357, 358], especially when targeting larger regions of damaged or diseased tissue. Furthermore, it is unlikely that simply bioprinting cell-laden constructs that mimic the shape of the meniscus will be sufficient; post-printing *in vitro* maturation to produce constructs that recapitulate the spatially defined structure, composition and biomechanics of the native tissue will also likely be required to develop functional and truly regenerative implants [223].

The identification of a suitable cell population for meniscus regeneration will be integral to satisfactory regenerative outcomes, and it is still a subject of ongoing debate. Primary meniscus cells have been shown to lose their native phenotype upon serial subculture, motivating increased interest in mesenchymal stromal/stem cells (MSCs) as an alternative cell source for meniscus tissue engineering [99, 101, 102, 106, 359]. Although MSC-based therapies for meniscus repair have shown some promise, their tendency to undergo hypertrophic differentiation and trigger mineralisation via endochondral ossification remains a major concern [103, 360]. Recently, the identification of a migratory and multipotent cell population within the inner and outer regions of the meniscus has increased interest in their isolation and use for meniscus tissue

engineering [109, 110, 115, 361]. Using a differential adhesion to fibronectin protocol that has been used to isolate progenitor cells from the articular cartilage [116, 117], it is possible to isolate cellular subpopulations from within the meniscus which have a high proliferative capacity and are able to maintain their differentiation potential after serial subculture, unlike non-fibronectin selected meniscus cells [113]. Phenotypically distinct progenitor cells have been found in both the inner and outer regions of the meniscus, which retain distinct capabilities to differentiate into inner and outer meniscus fibroblasts with limited evidence of hypertrophy [114]. Therefore, integrating such meniscus progenitor cells (MPCs) into advanced biofabrication strategies could potentially enable the engineering of functional meniscus constructs that mimic the spatial composition of the native tissue.

In addition to mimicking the spatial composition of the meniscus, functional meniscus tissue engineering will also require recapitulating the spatial structure of the native tissue, which is dominated by the circumferentially aligned collagen fibres that are integral to its biomechanical function [17]. Scaffold-free or self-assembly tissue engineering strategies represent one promising route towards the generation of anisotropic biomimetic meniscus constructs, particularly when combined with appropriate bioactive stimuli [5, 174, 178, 219, 291, 362]. Another approach to control collagen microarchitecture in engineered tissues has been via the use of aligned electrospun scaffolds, which serve as a template to direct the orientation of cells and subsequent ECM deposition [161, 238]. We have previously used melt electrowritten (MEW) scaffolds within a multiple-tool biofabrication platform to bioprint anisotropic meniscus-like tissues with user-defined collagen architectures [345]. By altering the aspect ratio of the pores (or ‘microchambers’) in these MEW scaffolds, it was possible to preferentially direct collagen deposition parallel to the long axis of these guiding structures. A 30-fold increase in compressive mechanical properties was observed over 4 weeks of *in vitro* maturation, resulting in the development of engineered tissues that mimicked the compressive properties of the native tissue. Scaling-up such biofabrication

platforms may potentially enable the engineering of large, anatomically defined meniscal grafts, especially if combined with cell types capable of generating the spatially distinct regions of meniscus. Furthermore, an inherent advantage of MEW as a fabrication technique is the lower polymer content and the high porosity of the scaffolds due to the small filament diameter that can be obtained, which may improve its *in vivo* regenerative potential [6, 193].

The aim of the study is to combine the biological potential of MPCs, isolated from the inner and outer regions of the meniscus, with a multiple-tool biofabrication strategy to engineer large (5 mm tall) wedge-shaped meniscus-like tissues with spatially defined structure and composition. Building on previous work reported in the literature [113, 114], we first sought to use a differential fibronectin adhesion protocol to isolate MPCs from the inner and outer zone of the meniscus, and then compare their capacity to generate phenotypically distinct meniscus tissue compared to meniscus fibrochondrocytes isolated from the inner and outer zone of the meniscus. We then attempted to bioprint fibrocartilage tissue with preferentially aligned collagen fibres by depositing MPCs obtained from either the inner (iMPCs) or outer (oMPCs) region of the meniscus into MEW scaffolds with anisotropic pore networks. Finally, we spatially deposited iMPCs and oMPCs into scaled-up MEW scaffolds with a view to engineering wedge-shaped meniscal tissues with regionally defined composition and structure.

6.2. Material and methods

Melt Electrowriting (MEW): MEW scaffolds with a fibre diameter of 15 μm were fabricated on a custom MEW printer built in house [313]. Briefly, PCL (50,000 MW, CAPA 6500D, Perstorp) was melted at a temperature of 80 $^{\circ}\text{C}$ around the syringe, increasing to 85 $^{\circ}\text{C}$ around the nozzle (21G). To extrude the polymer, a pressure of 0.6 bar with an initial voltage of 6 kV was applied at a distance of 6 mm from the grounded aluminium collector plate. With every layer, the voltage was increased by 0.0125 kV. Fibres were subsequently deposited according to a predesigned architecture, obtaining a

scaffold with a pore size/shape of 0.4 x 1.6 mm. For the wedge-shaped scaffold, the starting height was set at 8 mm with an initial voltage of 7 kV. To obtain the wedge-shape profile, at every 1 mm of build, the width in one of the axes was reduced by 2-3 mm (for a total of 5 layers), to achieve a final scaffold height of 5 mm (Figure 6.5B).

Isolation and Expansion of Mesenchymal Stromal/Stem Cells (MSCs): Porcine bone marrow-derived MSCs were obtained from the femur of a pig supplied by a local abattoir (male, approximately 4 months old). The bone marrow was collected under sterile conditions from the femoral shaft, and diluted with expansion medium (XPAN), consisting of hgDMEM supplemented with 10% v/v FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (all Gibco, Biosciences). After obtaining a homogenous suspension using a 16G needle and filtering through a 40-µm nylon mesh, cells were counted using trypan blue containing 3% acetic acid before plating 23×10^6 cells into T175 flasks for expansion. All expansion was performed at 5% pO₂, and using XPAN medium supplemented with 5 ng/ml FGF2 (Peprotech) seeding at a density of 875,000 cells per T175 flask. Chondrogenic, osteogenic and adipogenic differentiation assays were used to assess the tripotentiality of isolated MSCs.

Meniscus Cell Isolation and Fibronectin Selection of Meniscus Progenitor Cells (MPCs): Medial and lateral knee menisci were obtained from a 4-month old pig. Under sterile conditions, the menisci were rinsed twice with PBS containing 100 U/mL penicillin and 100 µg/mL streptomycin (both Gibco, Biosciences). After 30 min of pronase digestion (500 U/mL) at 37 °C, inner and outer regions were plated separately and minced into pieces ~ 1-2 mm³ in size. The tissue samples were then placed in a collagenase (collagenase type I, Gibco) solution (900 U/mL) for 3 h at 37 °C under constant agitation. When samples had fully digested, the suspension was filtered through a 0.4 µm porous membrane, centrifuged and resuspended in hgDMEM.

For the fibronectin coating, 100 mm petri dishes were coated with a 10 µg/mL fibronectin (Promocell) solution in PBS containing 1 mM MgCl₂ and 1 mM CaCl₂

overnight at 4 °C, as previously described [117]. Dishes were then blocked using a 1% bovine serum albumin (BSA) for 1 h at RT. Isolated cells from the inner and outer region of the meniscus were resuspended at 4,000 cells/mL and plated separately onto coated dishes for 20 min at 37 °C in hgDMEM, or into non-coated plates for expansion in XPAN medium with 5 ng/ml FGF2. After 20 min, the media in the coated plates was removed together with non-adherent cells and replaced with XPAN medium with 5 ng/ml FGF2.

Chondrogenic Differentiation in Pellet Culture: Pellets were formed by centrifuging 250,000 cells in 1.5 mL microtubes at 500 g for 5 min. Pellets were maintained in chondrogenic medium (CDM), which consisted of hgDMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (both Gibco), 100 µg/mL sodium pyruvate, 40 µg/mL L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 4.7 µg/ml linoleic acid, 1.5 mg/ml bovine serum albumin, 1× Insulin–Transferrin–Selenium (ITS), 100 nM dexamethasone, 2.5 µg/ml Amphotericin B (all from Sigma) and 10 ng/ml of human transforming growth factor-β3 (TGF-β3) (Peprotech). Cells were cultured at 5% pO₂ for 4 weeks with medium changes performed every two days.

Osteogenic Differentiation and Alizarin Red Staining: For osteogenic differentiation, 100,000 cells were plated to a 6-well plate and maintained for 2-3 days in XPAN medium. After achieving a confluency of 80%, 100 nM of Dexamethasone, 10 mM glycerol phosphate and 0.05 mM ascorbic acid (all from Sigma) were added to regular XPAN. After 21 days, the cell monolayer was washed twice with PBS before the addition of iced ethanol at RT for 10 min. After removing the ethanol, each well was washed first with PBS and then with distilled water. 2 mL of 1% of Alizarin Red (Sigma) was added and incubated for 1 min before rinsing with distilled water and imaging.

Biochemical Analysis: The biochemical content of the constructs was analysed after finishing the *in vitro* culture. Each construct was digested with papain (3.88 U/mL) in ultra-pure water containing 0.1 M sodium acetate, 5 mM L-cysteine–hydrochloride hydrate, 5 mM methylenediaminetetraacetic acid (EDTA) (all from Sigma–Aldrich). The

pH was adjusted to 6.5 using 38% HCl. Samples were incubated in papain solution at 60 °C, rotating at 10 rpm for 18 h. Immediately after digestion, the DNA content was quantified using the Hoechst 33258 dye assay with calf thymus DNA as standard (Merck) reading at 360 nm excitation and 460 nm emission. The amount of sGAG was quantified using the 1,9-dimethylmethylene blue (DMMB) dye-binding assay, with a chondroitin sulphate solution as standard (Blyscan) [314]. To exclude any background absorbance from the alginate, the pH of the DMMB was adjusted to 1.5. The 530/590 absorbance ratio was used to generate the standard curve and determine the sGAG concentration in the digested samples. Total collagen content was determined by measuring the hydroxyproline content using the dimethylaminobenzaldehyde and chloramine T assay, and a hydroxyproline/collagen ratio of 1:7.69 [273]. Briefly, samples were mixed with 100 µL of 38% HCl, and incubated at 100 °C for 18 h. After cooling, samples were centrifuge at 5000 g for 5 min, and let to dry at 50 °C for 48 h. Dried samples were then dissolved in 200 µL ultra-pure water. 2.82 % (w/v) chloramine T solution was added and incubated for 20 min at room temperature in the dark, before the addition of a 50 % (w/v) 4-(Dimethylamino) benzaldehyde solution (both Sigma) at 60 °C for 20 min. Hydroxyproline levels were estimated from the standard curve at a wavelength of 570 nm.

Histological Analysis: Constructs were fixed in 4% (w/v) paraformaldehyde, dehydrated in a graded series of ethanol and xylene baths, embedded in paraffin wax, sectioned at 8 µm using a microtome (Leica Microsystems), and affixed to microscope slides. The sections were stained with haematoxylin and eosin (H&E), alcian blue, picrosirius red and alizarin red. For immunohistochemistry antigen retrieval was carried out by an initial treatment with pronase (3.5 U/ml; Merck) at 37 °C for 25 min, followed by hyaluronidase (4000 units/ml; Sigma-Aldrich) at 37 °C for 25 min for collagen type I and type II. For collagen type X, the antigen retrieval method consisted of an initial treatment with pronase (35 U/mL; Merck) at 37 °C for 5 min, followed by chondroitinase ABC (0.25 U/mL; Sigma-Aldrich) at 37 °C for 45 min. Non-specific sites were blocked using a 10%

goat serum and 1% BSA blocking buffer for 1 h at room temperature. Collagen type I (1:400; ab138492; Abcam), type II (1:400; sc52658; Santa Cruz), and type X (1:300; ab49945; Abcam) primary antibodies were incubated overnight at 4 °C, followed by 20 min treatment using a solution of 3% hydrogen peroxide (Sigma-Aldrich). The secondary antibodies for collagen type I (1:250; ab6720; Abcam), type II (1:300; B7151; Sigma-Aldrich) and type X (1:500, ab97228; Abcam) were incubated for 1 h at RT. Samples were then incubated for 45 min with VECTASTAIN Elite ABC before treating them with ImmPACT DAB EqV (both from Vector Labs) at RT. Slides were then imaged using an Aperio ScanScope slide scanner, while sections stained with picosirius red were imaged using polarised light microscopy. Analysis on the fibre orientation and coherency was carried out using the OrientationJ plugin in ImageJ software [344].

Oxidised Alginate Preparation (OA): The alginate oxidation was performed as previously described [271]. Briefly, 1 g of alginate (MVG, Pronova Biopolymers) was dissolved in 90 mL of deionised water overnight at RT, and mixed with 10 mL of sodium periodate (Honeywell) to achieve a theoretical alginate oxidation of 4% under stirring in the dark at room temperature for 24 h (based on the molecular weight of the repeat unit, MW 198 g/mol). The oxidised alginate was then purified by dialysis against deionised water for 3 days (MWCO 3500 Dalton; Fischer), sterile filtered through 0.22 µm filter, and lyophilised. Further details can be found here [343].

Inkjet Bioprinting: Inkjet bioprinting technology (3D Discovery, RegenHU) was used to deposit a cell-alginate bioink (3×10^7 cells/mL in 3% (w/v) oxidised alginate). A single droplet was deposited into each well to yield 17×10^3 cells/microchamber (valve opening time of 600 µs, which yields droplets of 0.566 µL). Immediately after bioink deposition, a 45 mM CaCl₂ solution in hgDMEM was added to crosslink the alginate for 5 min at RT. The crosslinking solution was then removed and replaced by XPAN medium with 5 ng/ml FGF2. After a period of 24 h, all constructs were switch to chondrogenic

differentiation medium (CDM). Throughout the culture, the constructs were maintained at 5% pO₂.

Mechanical Testing: To investigate the mechanical properties of the engineered tissues the different groups were tested using a single column Zwick (Zwick, Rowell) with a 10 N load cell. Unconfined compression tests were carried out in a PBS bath as previously described [274]. The Young's modulus was defined as the slope of the linear phase of the resulting stress-strain curve during the ramp phase of the compression to 10% or 20% strain. The equilibrium modulus was defined as the stress divided by the applied strain at the end of the relaxation phase, while the dynamic modulus was defined as the average stress over the five cycles divided by the dynamic strain amplitude [191]. Uniaxial tensile tests were performed on rectangular sections of dimensions 5 x 30 mm as previously described [195]. The elastic modulus was calculated from the linear region of the stress-strain curve.

RNA Isolation and Quantitative Real-Time PCR: 5 weeks after *in vitro* culture samples were washed in PBS before snap freezing them until further process. RNA was isolated via Trizol method (Sigma-Aldrich) followed by chloroform extraction and isopropanol precipitation as per manufacturer's instructions. RNA was re-suspended in RNase free water, snap frozen in liquid nitrogen and stored at -80°C. Polymerase chain reaction (PCR) with a high-capacity cDNA reverse transcription kit (Thermofisher) was conducted to transcribe 500 ng of RNA from each sample into cDNA. The levels of gene expression were measured with real-time PCR (Applied Biosystems) using SYBR green master mix (Applied Biosystems) and porcine specific primers (Table 6.1). Relative expression was obtained by $2^{-\Delta\Delta C_t}$ method and normalised to the arithmetic average of 2 housekeeping genes 18S and B2M [318].

Table 6.1. Primer sequences for real-time PCR.

Gene name	Gene full name	Forward/Reverse
B2M	Beta-2-microglobulin	F:5' ACTGAGTTCACTCCTAACG 3' R:5' TGCAGCATCTTCATAATCTC 3'
18S	Ribosomal protein S18	F:5' CAACACCACATGAGCATATC 3' R:5' AGAAGTTCCAGCACATTTTG 3'
ACAN	Aggrecan	F:5' CACCCCATGCAATTTGAG 3' R:5' AGATCATCACCACACAGTC 3'
SOX9	SRY-box 9	F:5' GACTGCTGAATGAGAGCGAGA 3' R:5' GAAGATGGCGTTGGGAGAGAT 3'
COL1A1	Collagen type 1 alpha 1 chain	F:5' TAGACATGTTTCAGCTTTGTG 3' R:5' GTGGGATGTCTTCTTCTTG 3'
COL2A1	Collagen type II alpha 1 chain	F:5' CGACGACATAATCTGTGAAG 3' R:5' TCCTTTGGGTCCTACAATATC 3'
COL10A1	Collagen typeX alpha 1 chain	F:5' CCAACATCCAGAATCCATC 3' R:5' GTAGGTGTTTGGTATTGCTC 3'
TNMD	Tenomodulin	F:5' GCTAATTGTCCTATTTTGGGG 3' R:5' GAAAGTGTGTTCCATGTCTG 3'

Extrusion Bioprinting: Cell-laden bioinks were prepared by mixing a 5.3% (w/v) alginate solution and a 21.5% (w/v) gelatine solution (Gelatine type B; Sigma-Aldrich) with a cell suspension (everything in hgDMEM) to obtain a final solution of 3.5% (w/v) of alginate and 5% (w/v) of gelatine containing 30x10⁶ cells/mL. The bioinks were loaded into a syringe, and then printed using the 3D Discovery bioprinter (RegenHU) at 16 °C, using a pressure of 0.07 MPa, a translational speed of 4 mm/s and a 27G needle (Adhesive Dispensing Ltd). After printing, the constructs were incubated for 30 min in a bath of 45 mM CaCl₂ in hgDMEM, and then the cell-laden hydrogel were maintained in XPAN medium for 24 h before switching to CDM at 5% pO₂. Media exchange was performed twice weekly until the end of the 6-week culture period.

Live/Dead Imaging: Cell viability was assessed using the live/dead assay 7 days after the bioprinting process. Briefly, constructs were washed in PBS followed by incubation for 1 h in PBS containing 2 µM calcein acetoxymethyl (calcein AM) and 4 µM ethidium homodimer-1 (EthD-1) (both from Bioscience) for 1 h. Samples were then washed in PBS

before imaging with a Leica SP8 scanning confocal microscope excited at 494 nm and 528 nm, and read at 517 nm and 617 nm.

Bioreactor Culture: All large-scale constructs (wedge-shaped) were cultured in a custom-made bioreactor system previously described after a first week of static culture [363]. The constructs were continuously translated at a speed of 0.05 mm/s and an amplitude of 3 mm for the entire culture period. Constructs were cultured in static for the first week and switched to dynamic conditions for the remaining 5 weeks at 5% pO₂.

Rheological Assessment of the Bioinks: The rheological properties of the alginate-based bioink were evaluated using a rheometer (MCR 102, Anton-Paar) equipped with a Peltier element for temperature control. A plate-plate geometry with a diameter of 25 mm (PP25) was used in all the tests. The viscosity as a function of shear rate (0.1 to 1000 /s) was conducted at a constant temperature of 16 °C.

Scanning Electron Microscopy (SEM): For SEM imaging, samples were mounted on SEM pin stubs with carbon adhesive discs and coated with gold/palladium for 60 s at a current of 40 mA using a Cressington 208HR sputter coater. Imaging was carried out in a Zeiss ULTRA plus SEM.

Statistical Analysis: Statistical analysis was performed using GraphPad Prism software. Statistical differences were determined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or Student's t-test, where appropriate. Numerical and graphical results are displayed as mean ± standard deviation. Significance was accepted at a level of $p \leq 0.05$. Sample size (n) is indicated within the corresponding figure legends.

6.3. Results

6.3.1. Fibronectin adherent populations from the inner and outer regions of the meniscus

Porcine menisci were harvested and processed to obtain fibronectin adherent meniscus progenitor cells (MPCs) and non-selected meniscus cells (NS) from the inner and outer

regions of the tissue (Figure 6.1A). Their chondrogenic capacity was evaluated by pellet culture of 250,000 cells after passage 2 (P2) and 4 (P4). After 4 weeks of chondrogenic culture, pellets derived from inner NS (iNS) and inner MPCs (iMPCs) had an average diameter of 2.2 ± 0.2 mm and 2.1 ± 0.1 mm, respectively, while those derived from outer NS (oNS) and outer MPCs (oMPCs) had an average diameter of 1.5 ± 0.1 mm and 1.4 ± 0.1 mm respectively (Supporting Figure S6.1). All cell populations displayed a (fibro)chondrogenic phenotype, as evident from the histological analysis where strong staining for sGAG and collagen deposition was observed, with no evidence of pellet mineralisation (Supporting Figure S6.2). At earlier passages (P2), immunohistochemistry analysis revealed no major differences between MPCs and NS populations in terms of their fibrocartilage phenotype (Figure 6.1B). However, at higher passages (P4) iNS displayed a loss of phenotype, as evident by diminished type II collagen staining. On the other hand, iMPCs maintained their ability to secrete collagen type II with prolonged passaging (P4). No difference in the DNA content of pellets was observed across the different groups (Figure 6.1C). At P2, all cell populations displayed a comparable capacity to secrete sGAG. However, further subculture to P4 was associated with a reduced capacity for sGAG deposition, except for the oMPCs (Figure 6.1D). At P4, pellets generated using iNS contained the lowest levels of sGAG, indicating that serial subculture negatively impacted maintenance of its native phenotype; this was also observed in the alcian blue staining (Supporting Figure S6.2). At P4, pellets generated using iNS also contained the lowest levels of collagen (Figure 6.1E), further confirming a loss of phenotype in these cells with serial subculture. Such a reduction in collagen synthesis with cell expansion was not observed in iMPCs, which secreted almost twice the amount of collagen as iNS in pellet culture following

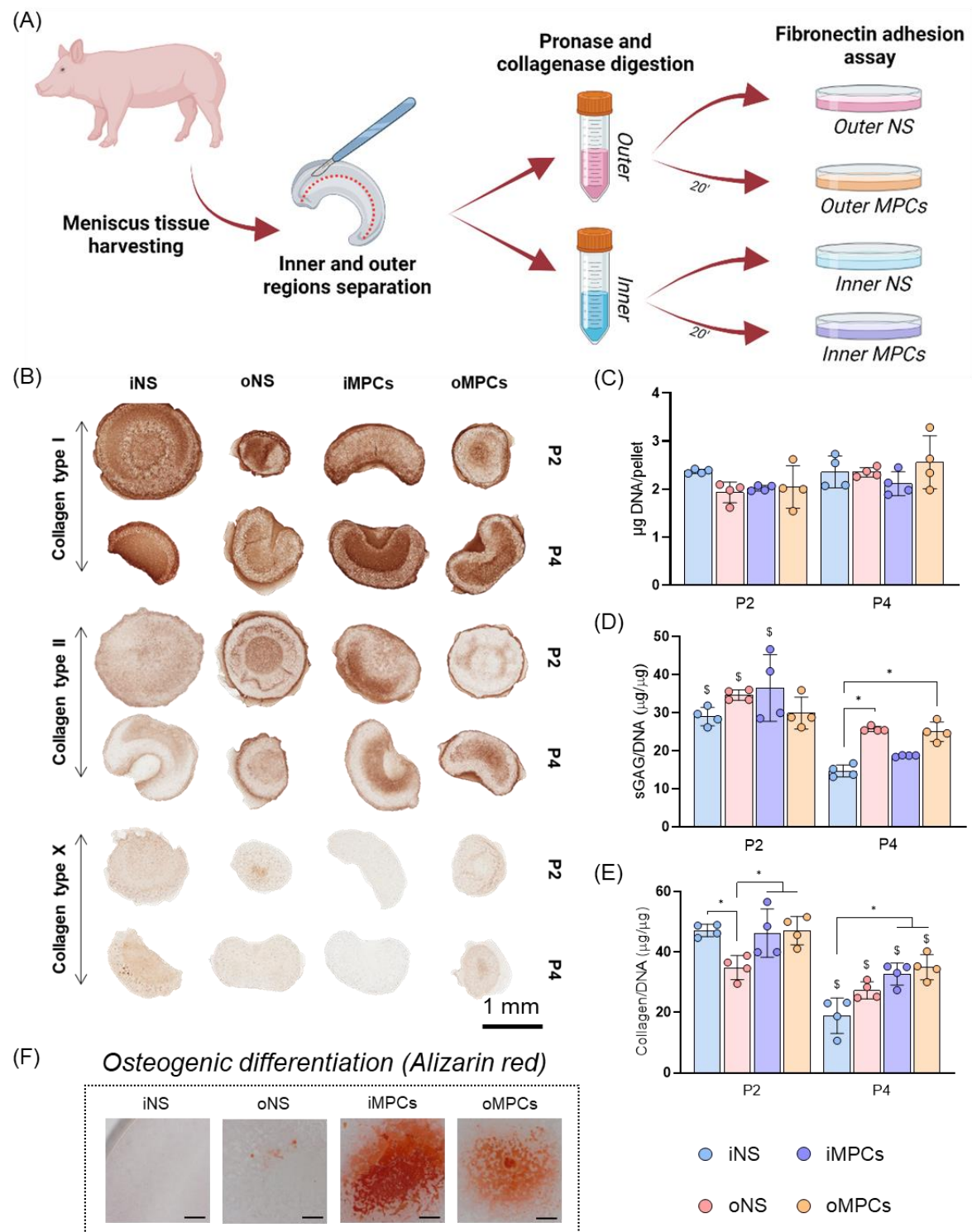


Figure 6.1. Chondrogenic and osteogenic potential of the different meniscus resident cell populations. (A) Schematic of the cell isolation process for non-selected (NS) and meniscus progenitor cells (MPCs). (B) Immunohistochemistry staining for collagen type I, type II and type X after 28 days of culture (scale bar is equal to 1 mm). Biochemical analysis at day 28 for the different cell populations, (C) DNA content, (D) sGAG content and (E) collagen content. All error bars denote standard deviation, significance was considered $*p \leq 0.05$, $n = 4$. \$ = significance between P2 and P4. (F) Alizarin red staining following 3 weeks of culture with osteogenic media. Scale bar is equal to 1 mm.

4 passages. Control MSCs isolated from bone marrow were found to secrete comparable levels of sGAG and collagen at P2 and P4 (Supporting Figure S6.3). NS did not demonstrate a capacity to undergo osteogenesis, with only MPCs generating an ECM that stained positive for mineral deposition following culture in osteogenic media (Figure 6.1F). It should be noted, however, that the osteogenic potential of MPCs was not comparable to bone marrow derived MSCs, which underwent more robust mineralisation *in vitro*. Overall, these results suggest that fibronectin selection can be used to isolate MPCs from the inner and outer regions of the meniscus; these cells maintain their differentiation capacity with prolonged monolayer expansion, opening their use within advanced biofabrication strategies.

6.3.2. Biofabrication of structurally organised and phenotypically distinct fibrocartilaginous tissues

After having demonstrated the potential of fibronectin selected MPCs, we next explored if our previously developed MEW PCL scaffolds could be used to engineer spatially organised fibrocartilaginous tissue using this cell source, similar to that observed using bone marrow derived MSCs [345]. To this end, MPCs and bone marrow derived MSCs were inkjet bioprinted into MEW PCL scaffolds with a pore (or microchamber) geometry of 0.4 x 1.6 mm, with a total height of 1 mm (Figure 6.2A). For these and subsequent experiments, we used cells at passage 4 unless otherwise stated. Building on previous investigations, a one-time cABC treatment was applied at day 14 of chondrogenic culture to enhance collagen network maturation [173].

Following 5 weeks of *in vitro* culture, robust chondrogenesis of MPCs was observed within the MEW scaffolds, as evident by alcian blue staining for sGAG deposition (Figure 6.2B). Small nodes of calcification were observed in the tissues generated using MSCs, which was not observed for the MPCs. MSCs, iMPCs and oMSCs all secreted abundant levels of sGAG, with MSCs secreting the highest levels of sGAGs

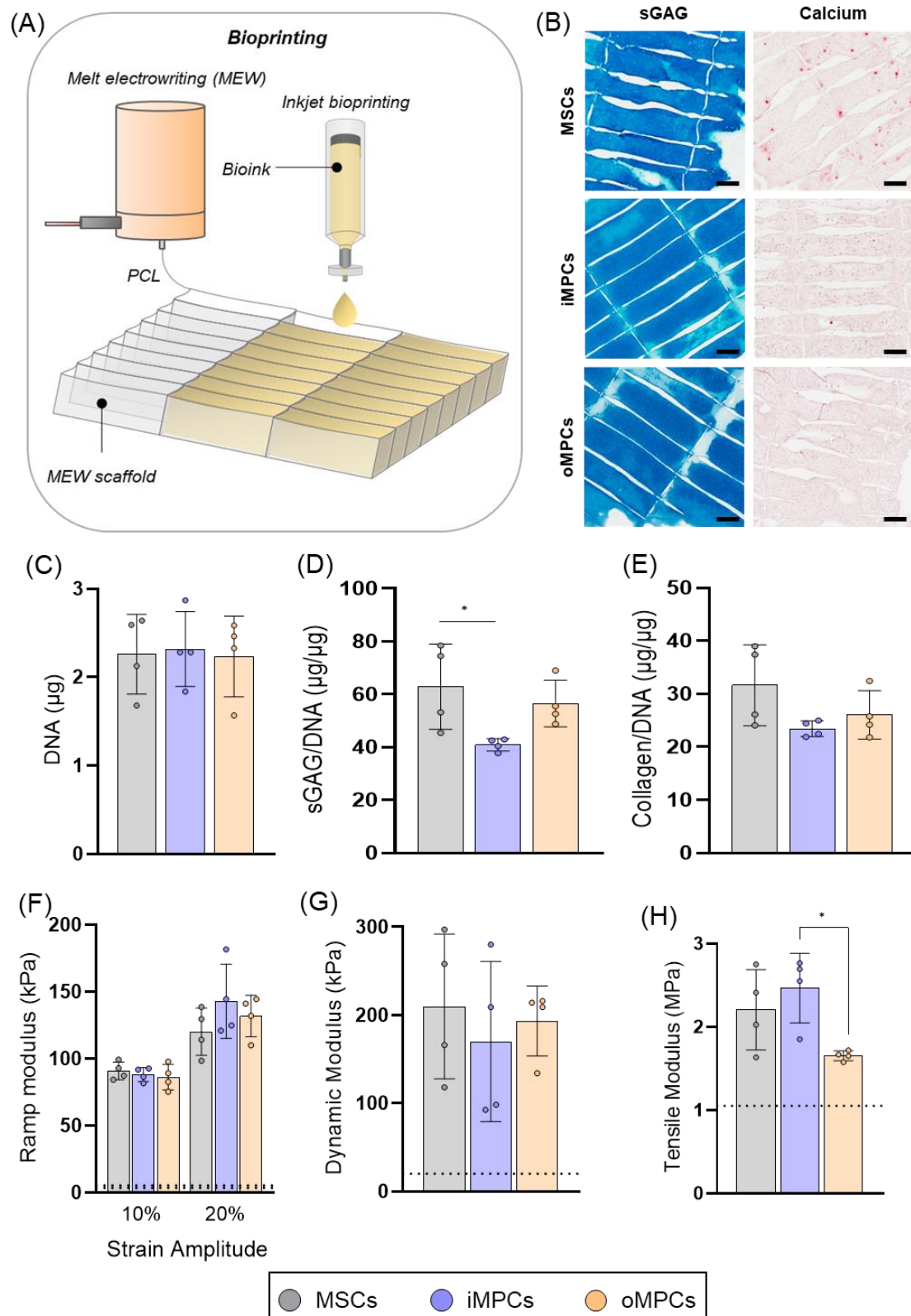


Figure 6.2. Histological analysis and biochemical content of the engineered tissues. (A) Schematic of the bioprinting process into the MEW scaffolds. (B) Histological staining for sGAG (Alcian blue) and calcium deposits (Alizarin red). Scale bar is equal to 400 μm . Biochemical analysis at day 35 for the different cell populations, (C) DNA content, (D) sGAG content and (E) collagen content. Biomechanical analysis at day 35 for the different cell populations (F) compression modulus of the constructs derived from the linear region of the compressive stress-strain curves, and (G) dynamic modulus. (H) Tensile modulus of the constructs derived from the linear region of the tensile stress-strain curves. Dashed lines represent empty MEW scaffold. All error bars denote standard deviation, significance was considered $*p \leq 0.05$, $n = 4$.

after the 5 weeks in culture (Figure 6.2D). All three cell populations deposited similar amounts of collagen (Figure 6.2E).

Next, the compressive and tensile properties of the engineered constructs were assessed [191]. No significant difference was observed in either the compressive ramp or dynamic modulus of the different constructs (Figure 6.2F, G), although all constructs were over 25 times stiffer than that of empty MEW scaffolds, with the ramp modulus values ranging between 75.24 and 99.20 kPa at 10% strain, and 98.40 and 181.5 kPa at 20% strain. Similar to native meniscus tissue, which displays dramatic tension-compression nonlinearity, all bioprinted tissues were over an order of magnitude stiffer in tension than in compression. This can be at least partially attributed to the high aspect ratio of the MEW fibres, which preferentially increases the tensile properties of the resulting construct. After 5 weeks of culture, the tensile stiffness of tissues bioprinted using iMPCs was higher than those generated using oMPCs (Figure 6.2H).

We next sought to investigate if preferentially aligned collagen was laid down by MPCs within the MEW scaffolds. Picrosirius red staining confirmed robust collagen deposition in all groups (Figure 6.3A). We then used polarised light microscopy (PLM) to analyse the degree of orientation of the collagen fibres in the engineered tissues. From the colour map, most of the fibres appeared to be oriented parallel to the long axis of the scaffold pores or microchambers, with a small portion of the fibres also oriented parallel

to the short axis. Further quantification indicated that most of the fibres were oriented

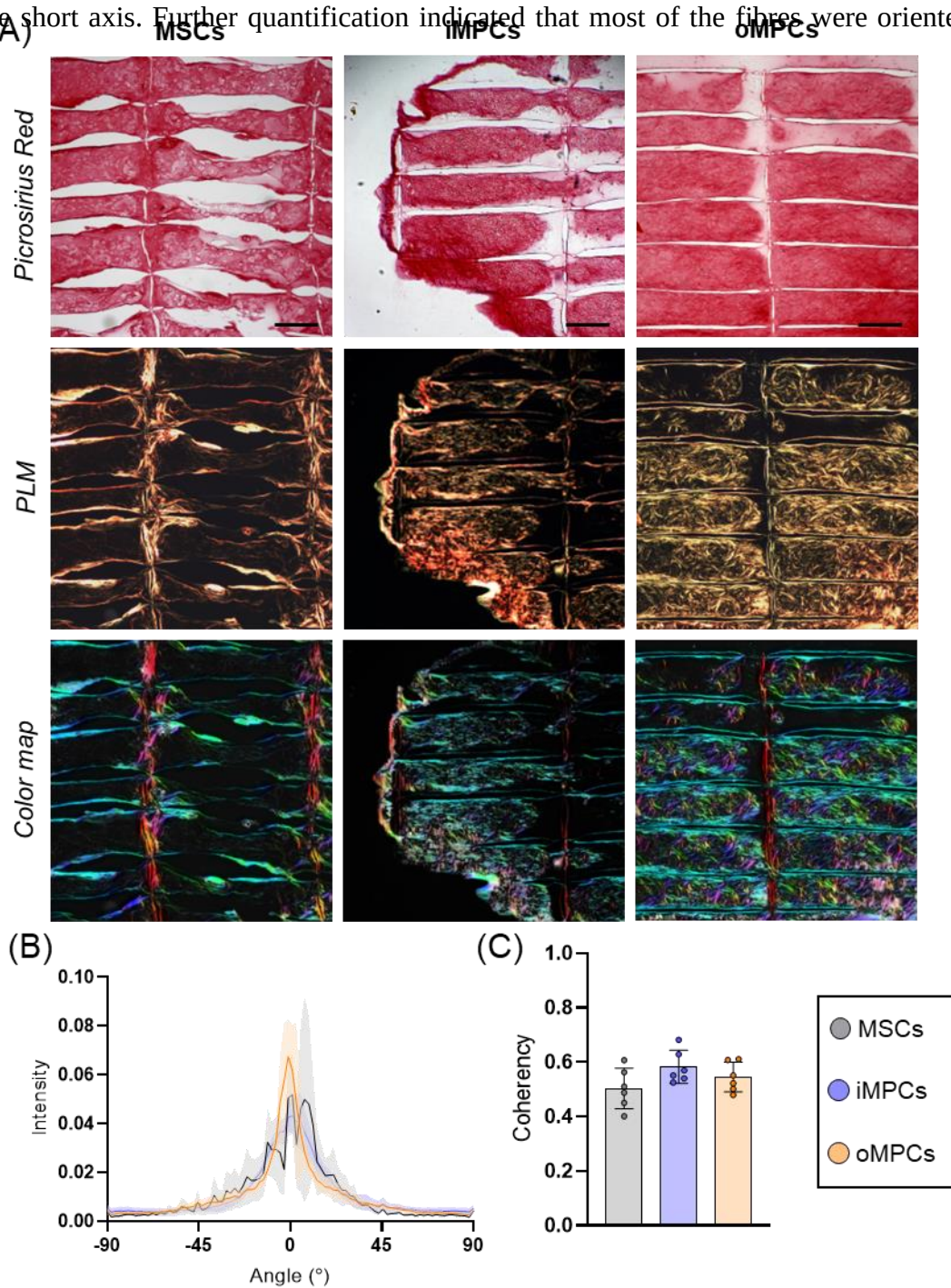


Figure 6.3. Collagen fibre orientations for the different cell populations following 5 weeks days of *in vitro* culture. (A) Picrosirius red, polarised light (PLM) and colour map imaging of the collagen fibre distributions. Scale bar is equal to 400 μm . Here cyan/blue colours denote fibres oriented at 0 degrees while purple/red denote fibres oriented at 90 degrees. (B) Quantification of the fibre orientation. (C) Quantification of the fibre coherency, where values approaching to 1 indicate that fibres are aligned towards a preferential direction. All error bars denote standard deviation.

preferentially at $0.1 \pm 0.1^\circ$, following the physical boundaries imposed by the MEW fibres. Furthermore, coherency values, which are a measure of the collagen fibres dispersion, confirmed a preferential organisation of the secreted collagen network (Figure 6.3C).

To characterise tissue phenotype, immunohistochemistry was undertaken to identify the collagen types present within the engineered tissues (Figure 6.4A). Tissues generated using oMPCs stained more intensely for collagen type I, but less intensely for collagen type II. Light staining for collagen type X was observed in all groups. qPCR confirmed that oMPCs express the lowest levels of type II collagen, adopting a phenotype more representative of the outer region of the meniscus, with a higher ratio of collagen type I to type II expression (Figure 6.4B-D). Tenomodulin expression, which is a known tendon marker expressed in the outer region of the meniscus, was also 8-fold higher in constructs generated using oMPCs compared to the other cell populations (Figure 6.4G). Conversely, the expression of type I collagen relative to type II collagen was lower in iMPC compared to oMPCs, indicating a more inner zone or hyaline-like cartilage phenotype (Figure 6.4D). Collagen type X expression was dramatically lower in the MPCs compared to MSCs. Aggrecan expression was also ~ 5 -fold higher in MSCs compared to the MPCs (Figure 6.4F). Taken together, these results indicate that the MPCs display a unique phenotype that resembles that of their native niche, making them a promising cell source for meniscus tissue engineering.

6.3.3. Biofabrication of scaled-up, wedged-shaped fibrocartilaginous tissues

The native meniscus has a unique wedge-shaped profile which is important for the stability of the knee joint. In order to recapitulate this anatomical feature, we 3D scanned a porcine meniscus to reconstruct its profile (Figure 6.5A). An iterative MEW process was implemented to print scaffolds with a similar ‘stepped’ profile (Figure 6.5B). This

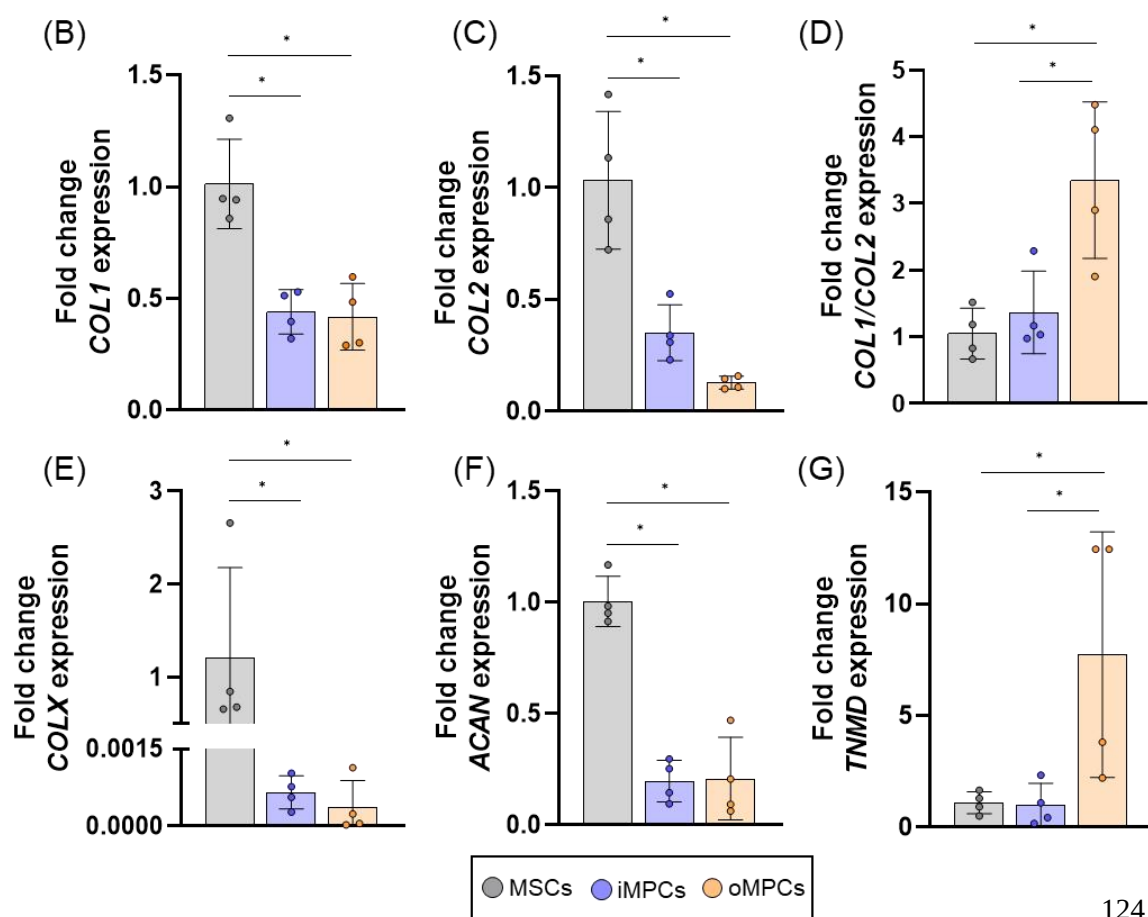
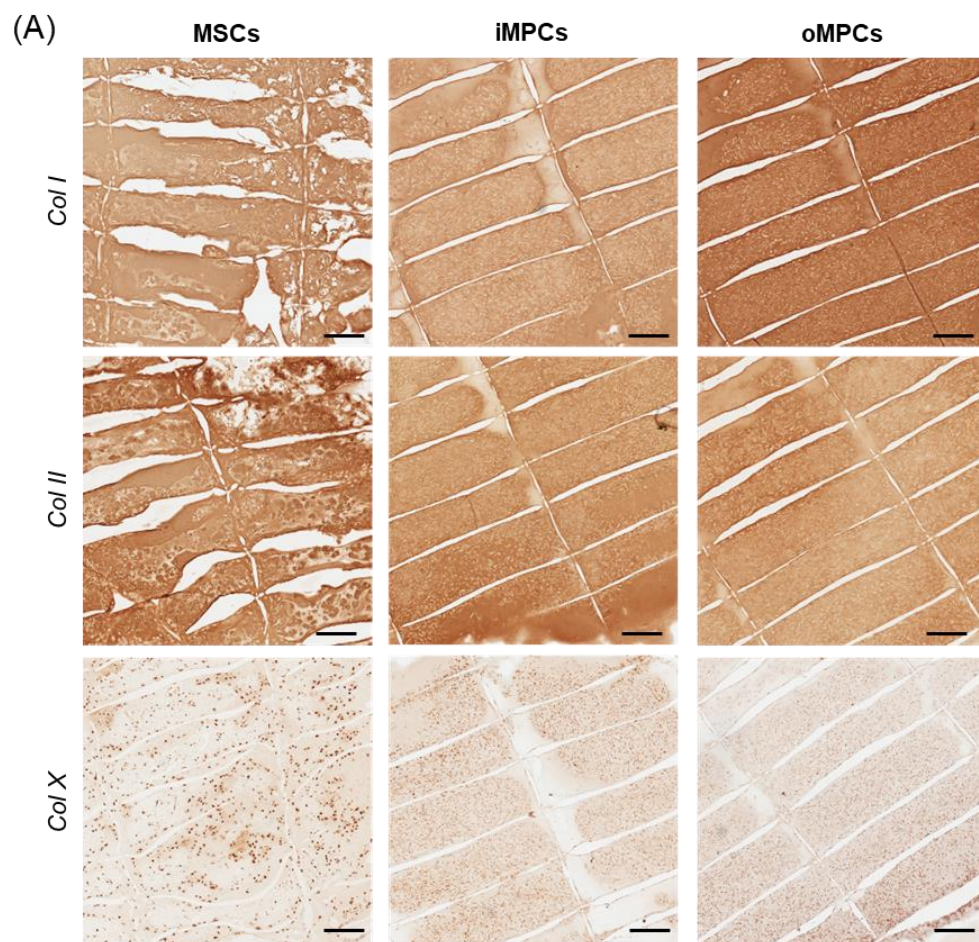


Figure 6.4. Phenotypical characterisation . A) Immunohistochemical staining for collagen type I (Col I), collagen type II (Col II) and collagen type X (Col X) deposition. Scale bar is equal to 400 μm . qPCR analysis of the engineered tissues using the different cell types, showing relative gene expression of (B) collagen type I, (C) collagen type II, (D) collagen type I/type II ratio, (E) collagen type X, (F) aggrecan and (G) TNMD, normalised against the housekeeping genes B2M and 18S. Statistically significant difference are marked with an * ($p \leq 0.05$).

process allowed us to print constructs of up to 5 mm height without any detrimental effect on scaffold porosity and interconnectivity (Figure 6.5B 1-4).

To spatially deposit MPC or MSC laden bioinks into these anatomically defined MEW scaffolds, we implemented an extrusion-based bioprinting process. Specifically, vertical extrusion (in the z-axis) allowed the controlled deposition of shear-thinning oxidised alginate-gelatine bioinks (Supporting Figure S6.4) into the pores of the MEW scaffold. To promote nutrient diffusion within such scaled-up constructs, sacrificial channels of gelatine (that melt at 37 °C) were also 3D printed in the construct; these left behind empty nutrient channels upon melting (Supporting Figure S6.5). Live/dead analysis 7 days after bioprinting confirmed excellent cell viability, indicating that the biofabrication approach could successfully be used for the engineering of large constructs (Figure 6.5C).

We next sought to engineer tissues with regionally distinct phenotypes resembling the native meniscus by spatially depositing iMPCs and oMPCs into the inner and outer regions of the scaled-up MEW scaffold. To that end we bioprinted two different constructs: (1) using only MSCs, and (2) using iMPCs and oMPCs into distinct regions of the MEW scaffold to engineer constructs more mimetic of the native meniscus (Figure 6.6A). One week after bioprinting, the different constructs were transferred to a custom bioreactor for another 5 weeks of culture under dynamic conditions. After a total of 6 weeks in culture, robust tissue formation was observed macroscopically throughout the scaffold (Figure 6.6B). Histological staining for total collagen and sGAG also demonstrated ECM deposition throughout the entire scaffold for both MSCs and MPC

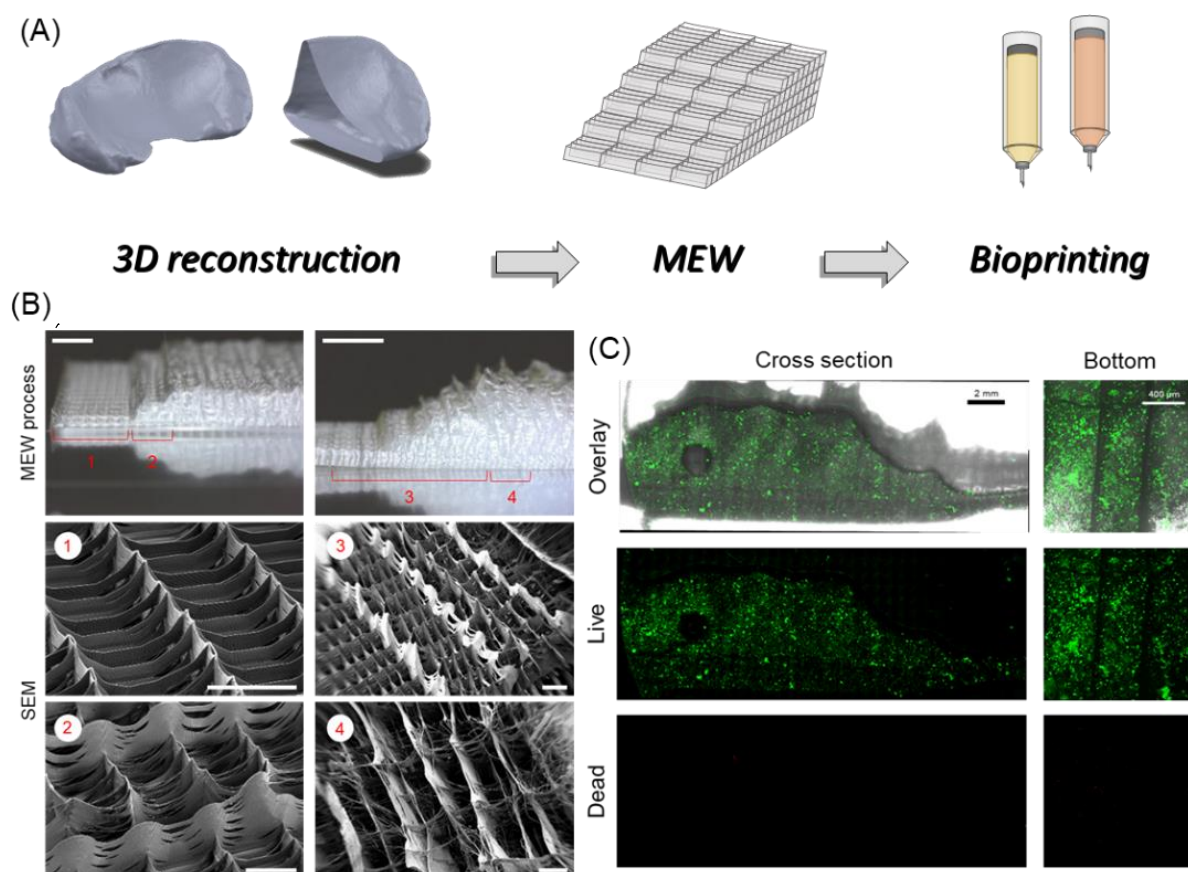


Figure 6.5. MEW fabrication of the wedged-shaped scaffold and bioprinting of cells. (A) Outline of the biofabrication process of wedge-shaped constructs. (B) Top – Macroscopic images of the melt electrospun scaffold during and post printing. Scale bar is equal to 4 mm. Bottom – Scanning electron microscopy images of different regions of the scaffold. (C) Cell viability (MSCs) in cross section and bottom plane after 7 days in culture. Scale bars are equal to 2 mm and 400 μm respectively.

groups, with little evidence of calcification (Figure 6.6C). Immunohistochemistry revealed a collagen type II rich hyaline cartilage-like matrix within the MSC constructs, whereas a more type I collagen rich fibrocartilage-like matrix was generated by the MPCs (Figure 6.6C). PLM confirmed that the collagen orientation followed the architecture of the MEW scaffolds pores/microchambers (Supporting Figure S6.6A-B), confirming that this physical guidance is not lost in our scaled-up tissues. Quantitative evaluation through biochemical assays demonstrated that there were no major differences in total levels of ECM production across the different groups (Supporting Figure S6.6C).

6.4. Discussion

This study demonstrates the capacity of MPCs to synthesise neo-fibrocartilage following prolonged passaging and to be used in the bioprinting of anisotropic and phenotypically defined meniscal constructs. These cells, isolated based on binding to fibronectin, maintained their region-specific meniscal phenotype upon serial expansion, expressing extracellular matrix components specific to their origin. By jetting zone-specific MPCs into MEW scaffolds with elongated pores, it was possible to engineer structurally organised meniscal tissue with significant tension-compression non-linearity. These meniscal-like tissues recapitulated key features of the inner and outer zone of the meniscus, including differential expression of collagen type I, collagen type II, and zone-specific markers such as tenomodulin (TNMD), further confirming the potential of zone-specific MPCs for the bioprinting of zonally defined meniscal grafts. Finally, we biofabricated a 5mm high MEW scaffold with a wedged-shaped profile similar to that of the native tissue, into which we spatially deposited iMPCs and oMPCs, thereby enabling the biofabrication of scaled-up meniscal constructs. Taken together, this work motivates (1) the use of MPCs for meniscus tissue engineering, and (2) the application of MEW in combination with bioprinting technologies for the biofabrication of meniscus-like tissues.

Based on differential adhesion to fibronectin [113-115, 118], we were able to isolate progenitor cell populations from both the inner and outer zone of the meniscus. MPCs maintained their capacity to generate zone-specific meniscal tissue upon serial passage, making them a particularly attractive cell source for meniscus TE strategies where large numbers of cells are required to fabricate human scale tissues. Unlike MSCs, which have an inherent tendency to become hypertrophic and progress along an endochondral pathway [103, 364], MPCs expressed type X collagen (a marker of hypertrophy) at very low levels, and the resultant tissues did not calcify in culture. Furthermore, while MPCs generate a calcified tissue in osteogenic culture conditions, they do so to a lesser extent than bone marrow derived MSCs. Zone specific MPCs also

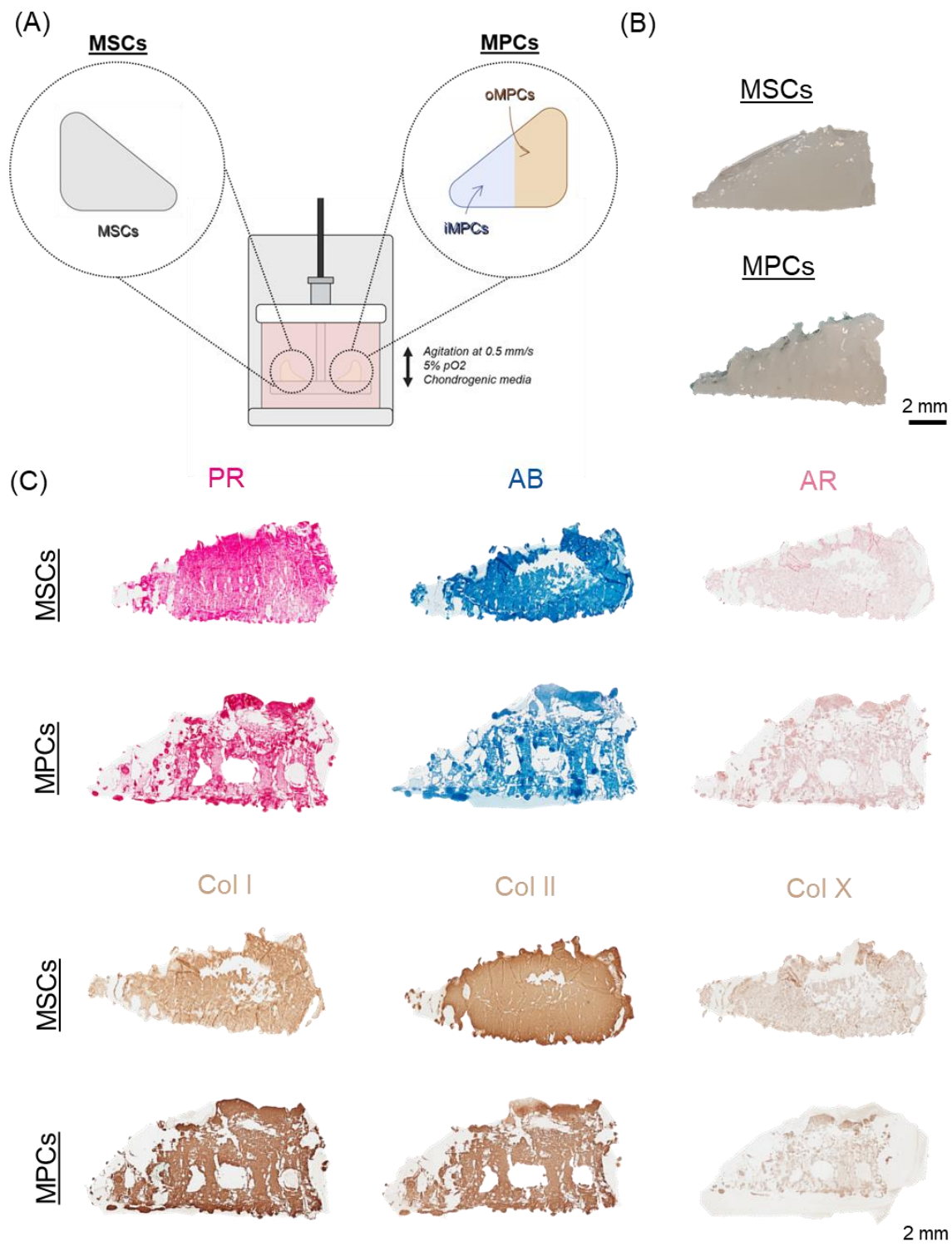


Figure 6.6. *In vitro* chondrogenesis within the wedge-shaped scaffold bioprinted with MSCs or MPCs and cultured in a dynamic culture system for 6 weeks. (A) Outline of the groups and the dynamic culture conditions. (B) Macroscopic images of the constructs following the 6 weeks of culture. Scale bar is equal to 2 mm. (C) Histological and immunohistochemical staining for total collagens (PR), sulphated glycosaminoglycan (AB), calcium deposition (AR), collagen type I (Col I), collagen type II (Col II) and collagen type X (Col X) deposition. Scale bar is equal to 2 mm.

appear superior to the use of inner or outer zone meniscal fibrochondrocytes for meniscus tissue engineering. Unlike non-selected fibrochondrocytes, which displayed a diminished capacity for ECM production with serial passaging, MPCs secreted comparable levels of sGAG and collagen at passage 2 and 4. It should be noted that the MPCs were isolated from animals that had not reached full skeletal maturity. Furthermore, changes in phenotype might be expected with skeletal maturation [312, 365], which might lead to the generation of MPCs with even greater zonal definition. A limitation of this study was that we did not undertake a detailed characterisation of the MPCs (e.g., flow cytometry) following the fibronectin isolation protocol. A recent investigation using fibronectin adhesion for the isolation of progenitor cells revealed that commonly used MSC-associated surface markers (e.g., CD90 and CD105) were not able to distinguish between selected and non-selected populations, indicating the need for more specific markers [111, 113]. Integrin marker expression levels has been suggested as potential target to identify progenitor cells in the meniscus tissue [118]. Future work is required to better characterise the progenitor cell populations isolated from the different regions of the meniscus, and to identify culture conditions that best maintain their phenotype during monolayer expansion. A limitation within the current work is the lack of data in population doublings since MPCs due to the selection process will inherently undergo through a larger number, what may hinder some differences between cell populations. Subsequent investigations will look into approaches to quantify the efficiency of the fibronectin selection process to provide a better estimation on the growth and proliferation of these different cell populations, and their effect on cell behaviour.

In addition to generating zone-specific meniscal tissue, MPCs were also able to generate structurally organised grafts in response to the physical constraints provided by the MEW scaffolds. We have previously shown that MSCs can generate such anisotropic tissues [345], and here we demonstrate that tissue-specific progenitor cells can also respond to such architectural cues and generate a collagenous matrix that aligns parallel to the long axis of the MEW scaffold pores. Such structured organisation has been

previously hypothesised to be directed by cell generated forces [366]. This results in collagen alignment that is dependent on the aspect ratio and width of the pores of the scaffold. The tensile and compressive properties of the tissues generated by the MPCs and MSCs were comparable, dramatically increasing over the 5-week culture period. Importantly, the resultant tissues displayed significant tension-compression nonlinearity, mimicking the mechanical behaviour of the native tissue. Despite this, the tensile stiffness of the engineered tissues was still significantly lower than that of the native tissue, where modulus values range between 100 and 200 MPa in the circumferential direction [44]. Surprisingly, the iMPCs generated a stiffer tissue than the oMPCs. This could be linked to differences in the relative production of type I and II collagens, and/or due to altered levels of sGAG accumulation which can negatively impact collagen network maturation [170-172]. Further work is required to improve the tensile mechanical properties of these grafts, which might involve the implementation of biochemical and biomechanical stimulation regimes which have shown to enhance the tensile properties of engineered tissues [8, 178, 181, 182].

The tissues generated following the jetting of iMPCs and oMPCs into the MEW scaffolds were also phenotypically distinct. Immunohistochemistry and gene expression analysis revealed oMPCs exhibit a higher Col I to Col II ratio compared to iMPCs and MSCs, thus mimicking the native collagen distribution [57, 367]. Furthermore, oMPCs expressed higher levels of TNMD, a marker more specific in the outer region of the meniscus, indicating meniscus derived cells retain regionally distinctive phenotypes [68]. The bioprinted tissues generated from both MPCs populations were also phenotypically distinct from that derived from MSCs. Significantly higher levels of GAGs were observed in chondrogenically primed MSCs, suggesting these cells are generating a more GAG-rich, hyaline cartilage-like tissue. Similarly, previous studies have shown that engineered tissues using meniscus-derived cell populations or MSCs not only differ in composition but also in terms of collagen matrix organisation, emphasising the need to adequately select the cell source for meniscus tissue engineering [172]. Together, these results point

to the potential of using iMPCs and oMPCs to biofabricate regionally defined constructs mimicking the zonal phenotypes observed in the native meniscus.

Having demonstrated the capacity to engineer anisotropic and phenotypically distinct fibrocartilaginous tissues using MPCs derived from the inner and outer regions of the meniscus, we next sought to biofabricate wedge-shaped meniscal tissues with regionally distinct compositions. Despite the significant advancements achieved by MEW in scaffold fabrication for tissue engineering, there remains a limitation in the maximum achievable scaffold height [206, 313]. In this study, a maximum height of 5 mm was reached while maintaining control over polymer deposition. Furthermore, the limited congruency of the scaffold described on this work could be overcome by using non-planar printing (a topic further described on Chapter 8). MEW is a relatively new technology that is undergoing continuous development and exploration, and it is only a matter of time before the current limitations of this technology are extended even further [368]. The application of z-printing enabled the rapid and controlled deposition of zone-specific MPCs within specific regions of the MEW scaffold [191, 221, 279]. Tissue engineering such scaled-up constructs requires satisfying the nutrient demands of large numbers of cells, which if not appropriately considered can lead to cell death and core regions devoid of matrix [363]. In order to address this challenge, we implemented the use of dynamic bioreactor culture and introduced microchannels into the bioprinted construct to support enhanced nutrient transfer. Dynamic bioreactor culture has previously been shown to enhance collagen and sGAG synthesis [221, 245, 369]. For the creation of microchannels for enhanced nutrient transfer, a fugitive gelatine-based bioink was deposited using z-bioprinting leaving behind a network of microchannels that supports nutrient transfer [370]. To generate regionally defined tissues, we deposited iMPCs within the inner region of the construct, and oMPCs within the outer region of a wedged-shaped scaffold. The MPCs were again found to generate a more fibrocartilaginous meniscal graft compared to MSCs, which instead supported the development of a more hyaline cartilage-like tissue rich in type II collagen. The incorporation of zone-specific biochemical and biophysical

cues might further enhance the development of regionally defined meniscal grafts in long-term culture [2, 3, 8, 91]. While these findings suggest that MPCs hold promise as a cell type for tissue engineering and regenerative medicine, further investigation is necessary to determine their full regenerative potential and clinical feasibility. The exploration of allogeneic use of meniscus progenitor cells is of great value, as it has the potential to enhance their applicability by increasing availability, reducing costs, and enabling cell preselection. Moreover, conducting comparative studies between MPCs and non-selected meniscus-derived populations for transplantation is crucial for advancing our understanding in this field.

6.5. Conclusion

In these series of experiments, we established a novel biofabrication framework that enabled the engineering of zone-specific meniscal tissue grafts. We first demonstrated the benefit of utilising meniscus progenitor cells over MSCs or meniscal fibrochondrocytes as a cell source for meniscus tissue engineering. We then demonstrated that it is possible to engineer structurally organised meniscal grafts, that displayed significant tension-compression nonlinearity, by inkjetting either iMPCs or oMPCs into structurally defined MEW scaffolds. Zone specific MPCs were able to generate tissues with distinct regional phenotypes following printing, including altered ratios of collagen type I to collagen type II expression, and differential expression of regional markers such as TNMD. Finally, we developed a bioprinting strategy to fabricate scaled-up wedge-shaped scaffolds using MEW. These findings encourage further investigation on the application of MPCs and MEW for the biofabrication of meniscal grafts for regenerative medicine applications.

Chapter 7: 3D bioprinting hydrogel boundaries to direct tissue organisation

7.1. Introduction

3D bioprinting has the potential to transform the fields of regenerative medicine and tissue engineering by enabling the precise deposition of cells and biomaterials and hence the engineering of complex biomimetic constructs [371]. Among the different bioprinting technologies, extrusion based bioprinting is the most widely used method, by which a bioink is deposited in the form of a continuous filament [123, 127]. This technology has been used to generate 3D structures recapitulating different features of native tissues, thereby enabling the manufacturing of regenerative implants and *in vitro* models [126]. In the field of orthopaedic tissue engineering, the ability to bioprint anatomically accurate cellular structures with spatial heterogeneity has enabled the biofabrication of constructs resembling articular cartilage and meniscus [52, 154, 257]. Such strategies commonly rely on homogeneously distributed cells within a hydrogel-based material, which are then matured *in vitro* in the presence of specific biochemical and/or biophysical cues, however the resulting tissues typically do not mimic the complexity of their native counterparts.

The different microarchitectures of biological tissues are associated with their unique mechanical behaviour and functionality [4, 312]. In the case of the meniscus, the tissue functions by distributing the loads throughout its circumferentially oriented collagen fibres [350, 372]. To date, the engineering of tissues that recapitulate the native collagen architecture remains a formidable challenge [38]. Many studies have shown how cells can sense, respond and migrate following mechanical, biochemical and geometrical cues [149, 223, 366, 373, 374]. Aligned nano- and microfibrous scaffolds can instruct and direct cell growth and phenotype, as well as subsequent tissue formation [161, 238].

Others, by taking advantage of the cellular contraction forces generated during the self-assembly process within ring-shaped agarose wells, were able to modulate collagen organisation leading to the development of anisotropic tissues [5]. The application of bioactive stimuli can then be used to further enhance the biomechanics of self-assembled meniscus-like tissues [170, 178, 181].

The Kelly lab has previously demonstrated that 3D printed porous polycaprolactone (PCL) structures can act as an effective method for driving the spatial organisation of self-organised tissues [221, 244, 245]. Over time, the PCL filaments acted as physical boundaries for the cells, which deposited a collagen network mimicking the arcade-like structure seen in native articular cartilage. In Chapter 4 of this thesis, it was demonstrated that highly elongated pore architectures in scaffolds fabricated using melt electrowriting (MEW) can be used to promote preferential alignment of the secreted collagen network, thereby mimicking aspects of the native meniscus [345]. A potential limitation of this approach is the use of PCL as a guiding biomaterial structure, as it degrades very slowly *in vivo* and mechanically might not be suitable within the knee joint environment (e.g., high stiffness, rapid plastic deformation). Physical cues provided within hydrogels, for example by controlling their porous architecture, have been shown to align cells locally and enable the development of organised tissue structures [369, 375]. While such moulding strategies are attractive, physical cues provided by 3D printing approaches may add the spatial control necessary to recreate the complex organisation of native tissues. These studies point to the potential of geometric boundaries as key regulators of engineered tissue organisation and associated mechanical functionality. Leveraging such know-how towards the bioprinting of functional tissues will require novel strategies to spatially and temporally control the physical boundaries applied to cells in bioprinted constructs.

Recent studies have pointed to the potential of 3D bioprinting as a platform to guide the process of cellular self-organisation, thereby enabling the engineering of large-

scale, highly complex tissues. Of note, it has recently been demonstrated that the bioprinting of high-density stem cells and organoids within a hydrogel matrix allowed the control over the self-organisation process, which followed the geometry and constraints imposed by means of bioprinting [246]. Those printed structures, following fusion of the cellular building blocks, were able to recapitulate native-like features such as lumens, branched vasculature and crypts from a millimetre to a centimetre scale. Another 3D printing strategy to introduce architectural cues within engineered tissues is the use of sacrificial inks embedded within a bulk hydrogel [247]. The embedding of these sacrificial templates within bulk hydrogels provided spatial and temporal control of the introduction of such architectural features by controlling the dissolution profile of the sacrificial ink.

By taking advantage of the controlled degradation of oxidised alginate (OA)-based bioinks, the aim of this chapter is to investigate if 3D bioprinting can be used to impose different geometrical boundaries which eventually can lead to direct and control the self-organisation of mesenchymal stromal/stem cells (MSCs) (Figure 7.1A). To this end this chapter will leverage two previously developed bioinks (in Chapter 3) [343]: (1) a fast degrading MSC-laden bioink (4% OA), which has been shown to dissolve within a short period (7 days); and (2) a slow degrading cell-free bioink (regular alginate), which will act as a physical boundary to confine the rapidly degrading bioink and provide geometrical cues to the printed cells. We hypothesise that the boundaries provided by the slow-degrading bioink will geometrically control the structural organisation of the resulting tissue.

7.2. Material and methods

Oxidised Alginate (OA) Synthesis: OA was prepared as previously described [271]. Briefly, 1 g of MVG sodium alginate (Pronova Biopolymers) was dissolved in 90 mL of ultrapure deionised water overnight at 37 °C, and mixed with 10 mL of sodium periodate (Honeywell) to achieve a 4% degree of theoretical alginate oxidation (Calculations were

based on that the molecular weight of the alginate repeating unit is 198 g/mol.). The solution was continuously stirred in the dark at room temperature (RT) for 24 h, followed by a dialysis step against deionised water for 3 days (MWCO 3500 Dalton; Fischer). The solution was then sterile filtered through 0.22 µm filter, after which it was lyophilised. The increased degree of oxidation was assessed through NMR spectroscopy at RT.

Isolation and Expansion of Mesenchymal Stromal Cells: Bone marrow derived MSCs were obtained from the femur of a 4-month old pig as previously reported [160]. Briefly, under sterile conditions, the bone marrow was removed from the femoral shaft, and mixed with expansion medium (XPAN), consisting of hgDMEM supplemented with 10% v/v FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (all Gibco, Biosciences). A homogenous suspension was achieved by triturating with a 16G needle. The suspension was then filtered through a 40 µm nylon mesh. Cells were counted and seeded into dishes for colony forming assay, or into T175 flasks for expansion. All expansion was conducted in hypoxic conditions at 5% pO₂, and using XPAN medium containing 5 ng/mL FGF2 (Peprotech) [272]. The tripotential of isolated MSCs was assessed using chondrogenic, osteogenic and adipogenic differentiation assays. Cells of passage 3 were used for experiments.

3D Bioprinting Process: Cell-laden bioinks were prepared by mixing a 5.3% (w/v) alginate solution and a 21.5% (w/v) gelatine solution (Gelatine type B; Sigma-Aldrich) with a cell suspension (everything in hgDMEM) to obtain a final solution of 3.5% (w/v) of alginate and 5% (w/v) of gelatine containing 20 or 60×10⁶ cells/mL. For acellular characterisation, the volume of cell suspension was substituted for hgDMEM to maintain the same polymer concentrations. The different bioinks were loaded into a syringe, and then printed using the 3D Discovery bioprinter (RegenHU) after leaving the bioinks for 10 min at 4 °C, using a pressure of 0.075 MPa, a translational speed of 6 mm/s and a plastic conical needle of 25G (Adhesive Dispensing). After printing, the different samples were incubated for 30 min in a bath of 45 mM CaCl₂ in hgDMEM, and then the cell-laden

hydrogels were maintained in XPAN medium for 24 h before switching to chondrogenic medium (CDM) which consisted of hgDMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (both Gibco), 100 µg/mL sodium pyruvate, 40 µg/mL L-proline, 50 µg/mL L-ascorbic acid-2-phosphate, 4.7 µg/mL linoleic acid, 1.5 mg/mL bovine serum albumin, 1× Insulin–Transferrin–Selenium (ITS), 100 nM dexamethasone, 2.5 µg/mL Amphotericin B (all from Sigma) and 10 ng/mL of human transforming growth factor-β3 (TGF-β3) (Peprotech) at 5% pO₂. Media exchange was performed twice weekly until the end of the 4-week culture period.

Live/Dead imaging: Cell viability was assessed using the live/dead assay. Briefly, constructs were washed in phenol-free DMEM (pfDMEM; Sigma-Aldrich) followed by incubation for 1 h in pfDMEM containing 2 µM calcein acetoxymethyl (calcein AM) and 4 µM ethidium homodimer-1 (EthD-1) (both from Bioscience) for 1 h. Samples were then washed in pfDMEM before imaging with a Leica SP8 scanning confocal microscope excited at 494 nm and 528 nm, and read at 517 nm and 617 nm.

Biochemical analysis: The biochemical content of the constructs was analysed after 4 weeks of *in vitro* culture. After washes with deionised water, each construct was digested with papain (3.88 U/mL) in ultra-pure water containing 0.1 M sodium acetate, 5 mM L-cysteine–hydrochloride hydrate, 5 mM methylenediaminetetraacetic acid (EDTA) (all from Sigma–Aldrich). The pH was adjusted to 6.5 using 38% HCl. Samples were incubated in papain solution at 60 °C, rotating at 10 rpm for 18 h. Immediately after digestion, the DNA content was quantified using the Hoechst 33258 dye assay with calf thymus DNA as standard (Merck) reading at 360 nm excitation and 460 nm emission. The amount of sulphated glycosaminoglycan (sGAG) was quantified using the 1,9-dimethylmethylene blue (DMMB) dye-binding assay, with a chondroitin sulphate solution as standard (Blyscan) [314]. To exclude any background absorbance from the alginate, the pH of the DMMB was adjusted to 1.5. The 530/590 absorbance ratio was used to generate the standard curve and determine the sGAG concentration in the digested

samples. Total collagen content was determined by measuring the hydroxyproline content using the dimethylaminobenzaldehyde and chloramine T assay, and a hydroxyproline/collagen ratio of 1:7.69 [273]. Briefly, samples were mixed with 100 μ L of 38% HCl, and incubated at 100 °C for 18 h. After cooling, samples were centrifuge at 5000 g for 5 min, and let dry at 50 °C for 48 h. Dried samples were then dissolved in 200 μ L ultra-pure water. 2.82 % (w/v) chloramine T solution was added and incubated for 20 min at room temperature in the dark, before the addition of a 50 % (w/v) 4-(Dimethylamino) benzaldehyde solution (both Sigma) at 60 °C for 20 min. Hydroxyproline levels were estimated from the standard curve at a wavelength of 570 nm.

Histological analysis: Constructs were fixed in 4% (w/v) paraformaldehyde, dehydrated in a graded series of ethanol and xylene baths, embedded in paraffin wax, sectioned at 8 μ m using a microtome (Leica Microsystems), and affixed to microscope slides. The sections were stained with picosirius red. Slides were then imaged using an Aperio ScanScope slide scanner and polarised light microscopy. Analysis on the fibre orientation and coherency was carried out using the OrientationJ plugin in ImageJ software [344]. The alginate samples were depolymerised using a citrate buffer (150 mM sodium chloride, 55 mM sodium citrate, and 20 mM EDTA in H₂O) for 60 min at 37°C under constant and gentle agitation [279].

Mechanical testing: To investigate the mechanical properties of the engineered tissues the different groups were tested using a single column Zwick (Zwick, Rowell) with a 10 N load cell. Unconfined compression tests were carried out in a PBS bath as previously described [274]. The Young's modulus was defined as the slope of the linear phase of the resulting stress-strain curve during the ramp phase of the compression to 10% strain. Uniaxial tensile tests were performed into rectangular section of dimensions 5 by 15 mm as previously described [195]. The elastic modulus was calculated from the stress-strain curves in the linear region.

Statistical analysis: Statistical analysis was performed using GraphPad Prism software. Statistical differences were determined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Results are displayed as mean $\pm$ standard deviation. Significance was accepted at a level of $p \leq 0.05$. Sample size (n) is indicated within the corresponding figure legends.

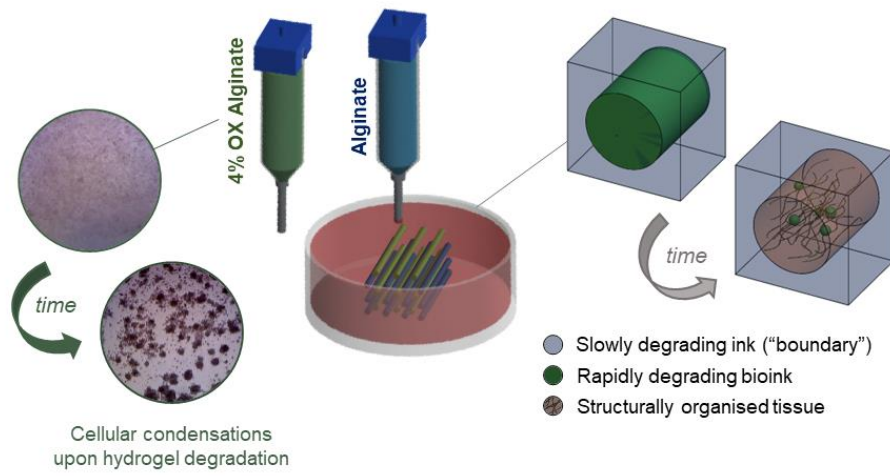
7.3. Results

7.3.1. Tailoring the self-assembly of cells within 3D bioprinted hydrogels

It was initially hypothesised that by printing cells in a fast-degrading alginate-based bioink, and then spatially confining these structures with regular or “slow” degrading bioinks, that it would be possible to direct the process of cellular self-organisation and print structurally organised tissues (Figure 7.1A). As a proof of concept, three simple designs were bioprinted; these were termed: (1) lines, (2) cylinder, and (3) square (Figure 7.1B). By using food colorant, it was possible to macroscopically visualise the spatial organisation of the different bioinks. Live/dead imaging after printing demonstrated that cells could also be patterned in the same fashion with good cell viability. Follow up images using live/dead showed that cells were able to self-organise within the imposed alginate boundaries within the printed construct. However, as cellular condensation progressed, it was not possible to maintain the initial printed geometry, with gaps emerging in the cell laden structures.

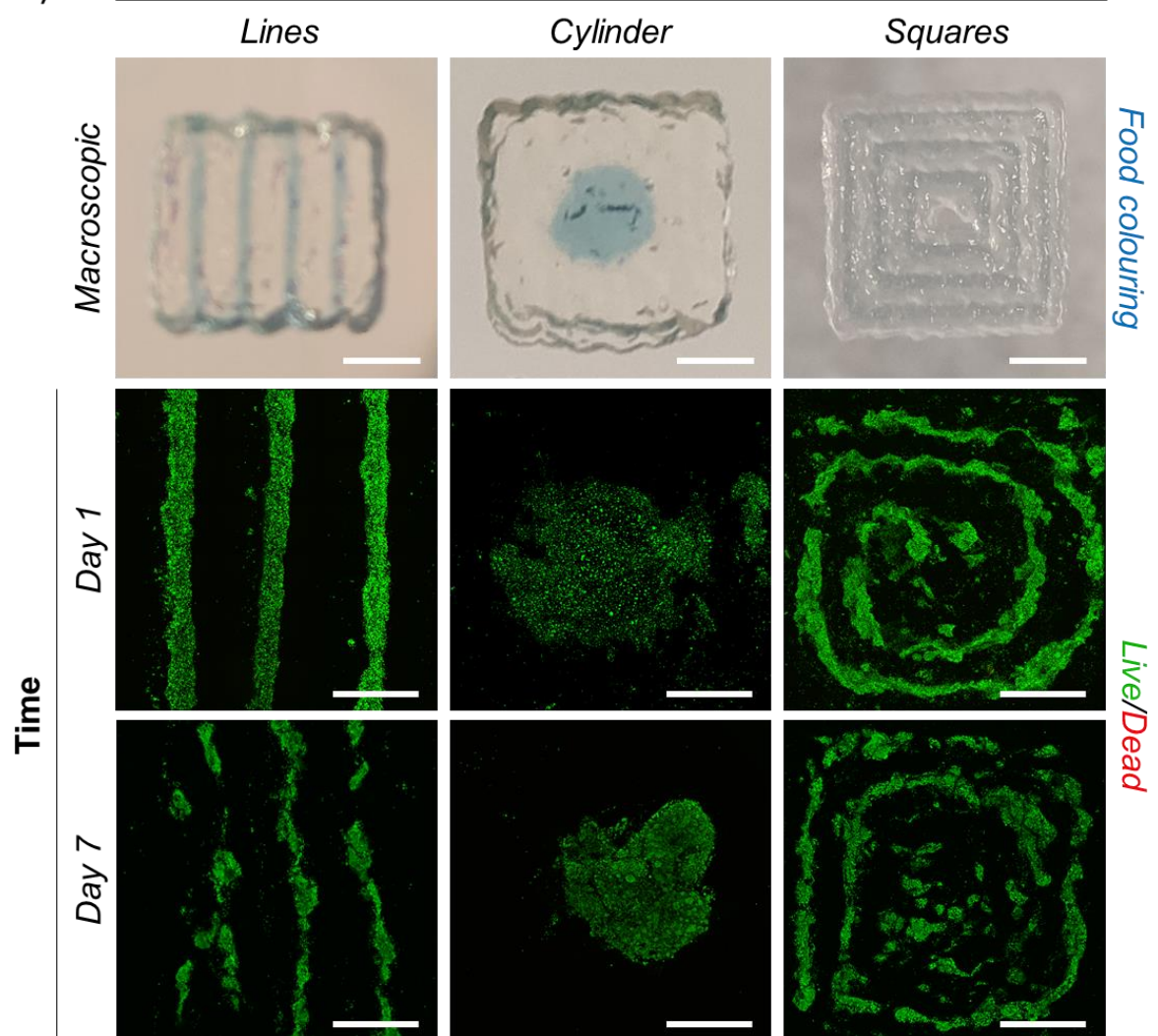
Figure 7.1. A, Illustration of the temporal boundaries concept using alginate boundaries to promote the directionality of the tissue. B, Top – Macroscopic images showing the control over the desired confinement geometry (oxidised alginate formulation supplemented with food colouring for visualisation). Scale bar, 3 mm. Bottom – Representative images of viability of MSCs after 1 and 7 days in culture post printing. Scale bar, 2 mm.

A)



B)

Design



7.3.2. Chondrogenic differentiation within the patterned hydrogels

Having demonstrated the ability to spatially localise and confine high concentrations of MSCs in 3D space, and their subsequent capacity to self-organise within the space defined by the slowly degrading inks, this chapter next sought to analyse the chondrogenic capacity of the bioprinted cells. In order to investigate the importance of the slow degrading ink as a boundary, and its role maintaining the geometrical stability of the bioprinted tissue, cellular constructs were fabricated with or without the confining alginate boundaries (Figure 7.2). Following 21 days of *in vitro* culture, matrix deposition could be seen macroscopically within all of the constructs. Importantly, without the presence of the confining alginate boundary walls, the rapidly degrading cell-laden bioinks lost their initial shape, forming strings of unorganised cartilage-like tissue. In

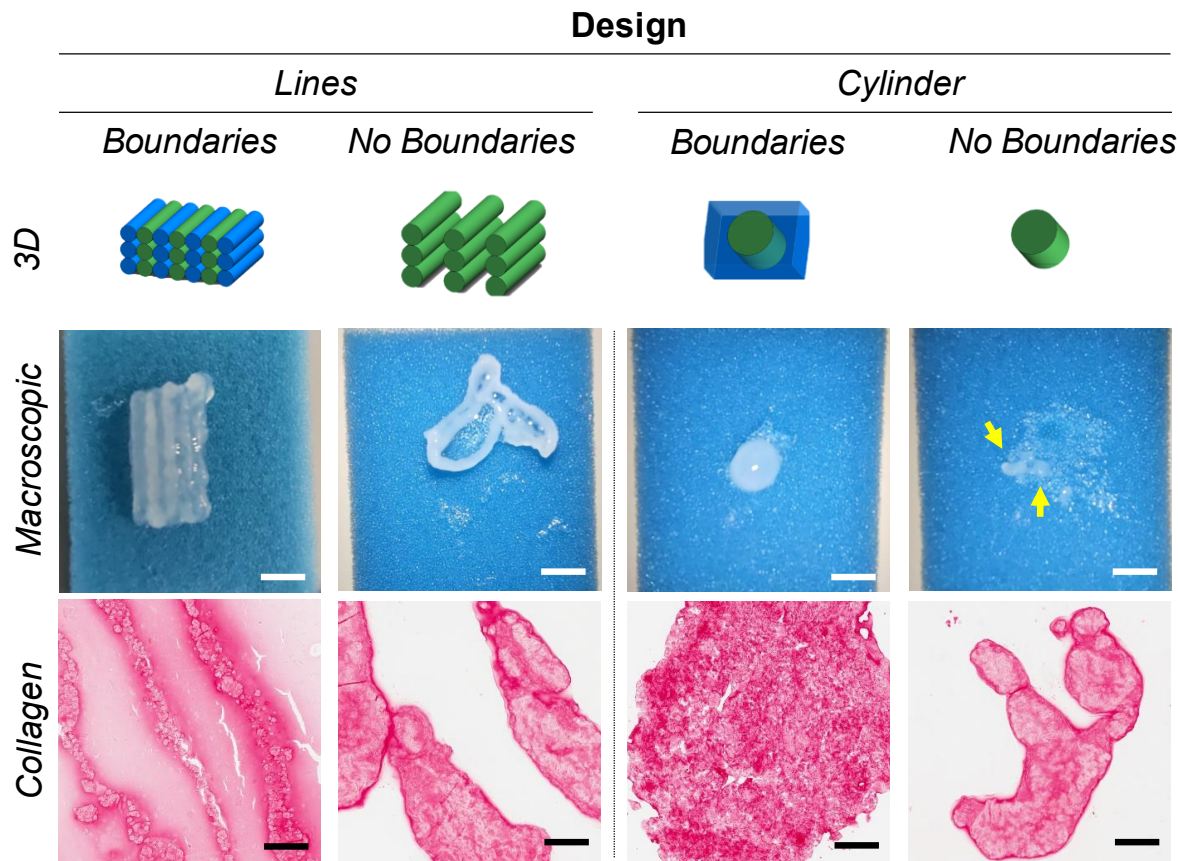


Figure 7.2. Effect of the use of boundaries. By applying boundaries (left) to rapid degrading bioinks (no boundaries) we can maintain the shape and form cartilaginous tissues with the original printed design. Scale bars from the macroscopic images are equal to 4 mm, while 500 μm for the histological images.

contrast, when confined (boundaries) by slowly degrading inks, the final shape of the self-organising tissue could be controlled. The ‘Lines’ of bioprinted cells generated stiff filaments of cells and extracellular matrix, while the ‘Cylinder’ of bioprinted cells maintained its shape. As a proof of concept, we removed the alginate component by using a citrate buffer at the end of culture, leaving behind only the cellular portion of the construct (macroscopic image on Figure 7.2). Supporting Figure S7.1 shows the same construct prior to the depolymerisation of the alginate.

7.3.3. Bioprinted architecture influence cell behaviour and resulting mechanical properties

Recognising that conventional cell encapsulation within hydrogels fail to recapitulate the complex microarchitecture of native tissues, this chapter next sought to investigate whether it was possible to direct the spatial organisation of the tissue generated by cells that are spatially confined by slowly degrading inks.

To this end, bioprinting parameters were varied in order to obtain three different boundary designs named (1) Alginate, (2) Aligned and (3) Offset (Figure 7.3A). The Alginate design served as a control, representing conventional approaches where cells are combined with a bioink and printed following a predesigned geometry. For the Aligned and Offset geometries, cells were encapsulated within the rapid degrading bioink, while the slow degrading bioink was used as a physical boundary. The Offset architecture differed from the Aligned due to the presence of an offset boundary layer at every other printed layer. Since the cells within the Aligned and Offset geometries would be confined within half of the printed construct (only on the rapid degrading bioink), the control was printed with half of the cell density in the bioink so that the final cell density per volume of construct would remain constant (Figure 7.3B). Following 28 days of *in vitro* culture, the Offset group contained the lowest sGAG and collagen content (Figure 7.3C, D). The mechanical properties in compression and tension were also assessed (Figure 7.3E, F).

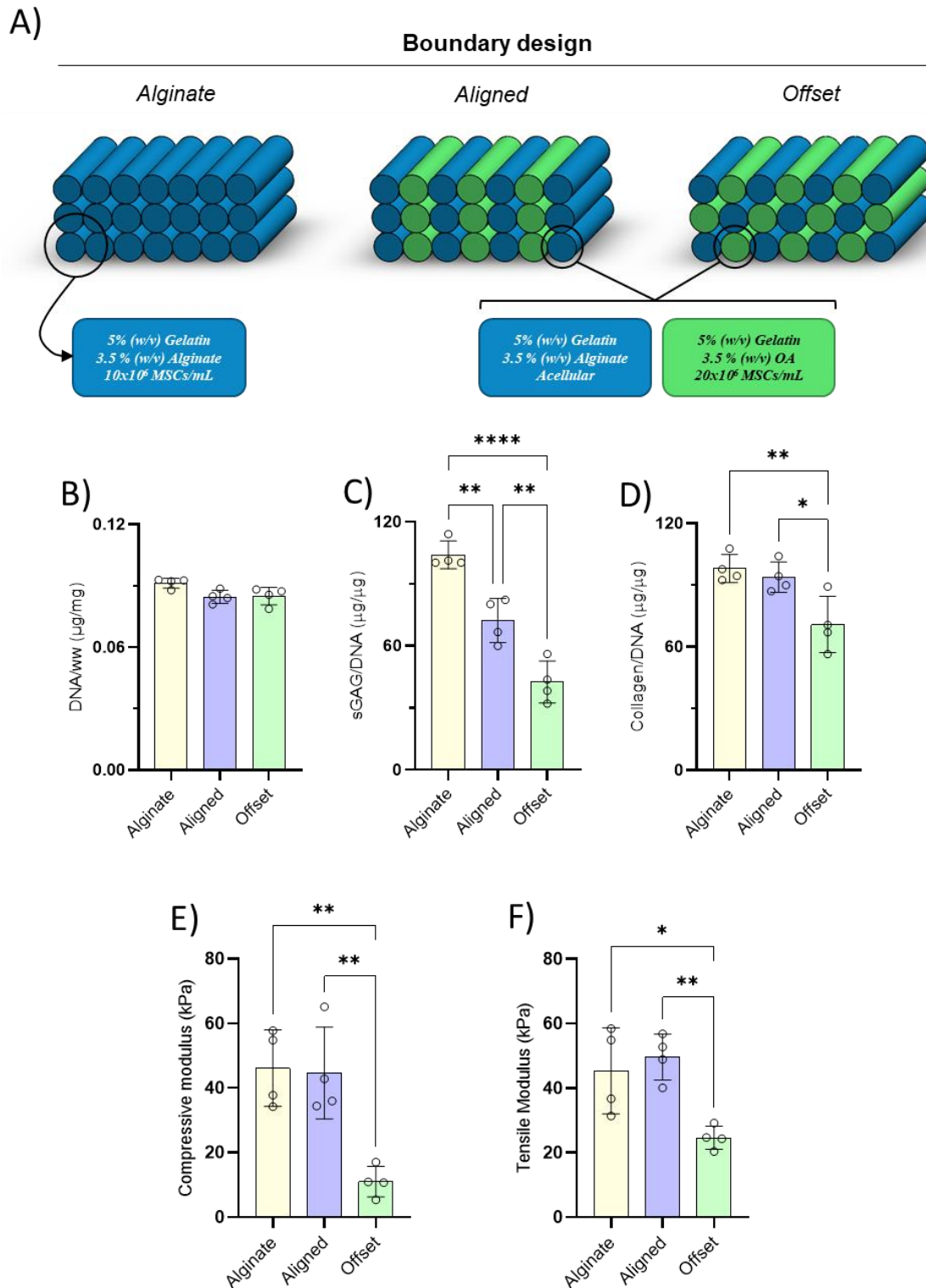


Figure 7.3. A, Illustration of the designs investigated (Alginate, Aligned and Offset). Quantification of (B) DNA, (C) sGAG and (D) collagen deposition following 28 days in culture. E, F, Compressive and tensile properties of the different designs after 28 days of in vitro culture. All error bars denote standard deviation, n = 4, significance was considered * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$.

The compressive modulus of the Alginate and Aligned groups were 46.15 ± 11.88 and 44.61 ± 14.20 kPa respectively, reducing to 10.99 ± 4.77 kPa for the Offset group. A similar trend was then found for the tensile properties, with the Aligned (49.65 ± 7.13 kPa) and Alginate (45.36 ± 13.33 kPa) groups significantly higher than the Offset group (24.64 ± 3.6 kPa).

7.3.4. Towards controlling the collagen organisation within bioprinted tissues

In order to study how these different boundary conditions influenced collagen alignment within the engineered tissues, we next performed polarised light microscopy (PLM) to elucidate the emerging collagen architecture. Representative images following 28 days of *in vitro* culture are shown in Figure 7.4. Although all the samples displayed strong staining for total collagens, their visualisation under PLM did not show a strong birefringence signal, with a clear signal only observed around the pericellular region. Interestingly, the boundaries within the Offset group had diffused and cells had spread

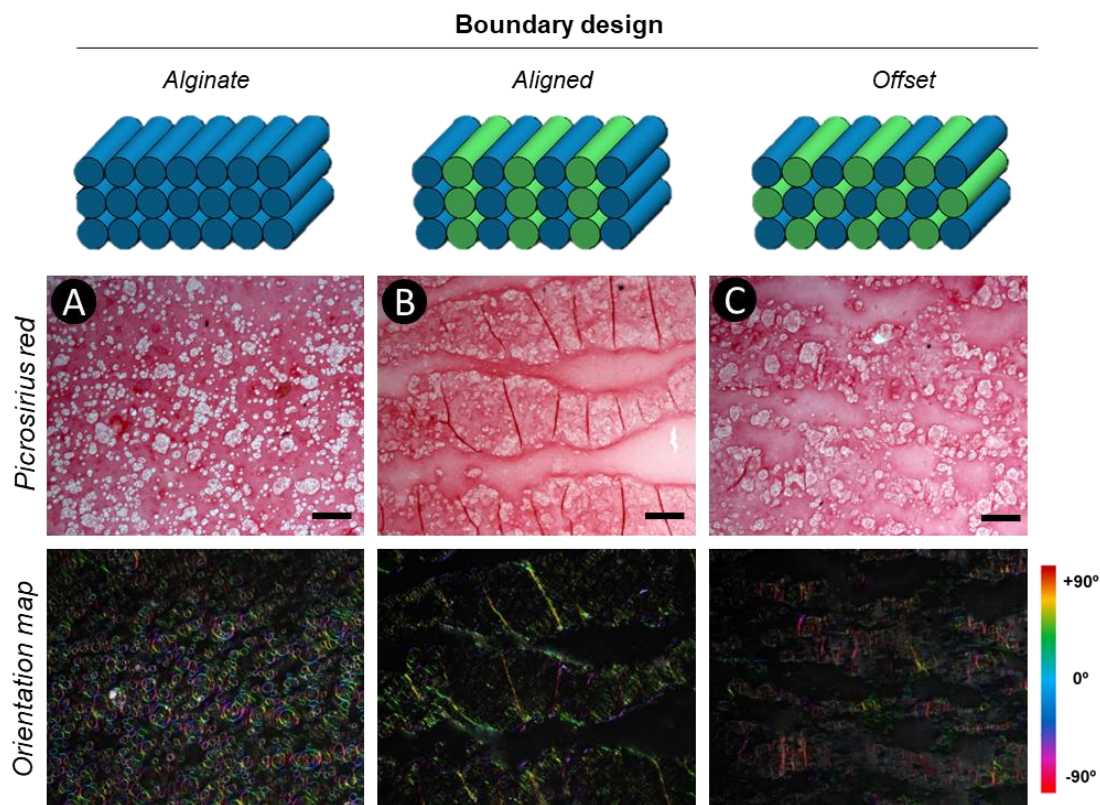


Figure 7.4. Top - Representative sections stained with picosirius red (total collagen). Bottom – Overlapping fibre orientation colour map of the collagen network. Colour wheel is provided to show how hue correspond to an orientation. A, Aligned; B, Alginate; C, Offset. Scale bar is equal to 400 μm .

around the hydrogel matrix, while within the Aligned design cells were still physically confined following the initial pattern.

Because cell density is a known factor influencing collagen fibrillation, we next aimed to investigate how increasing the cell density from 20 to 60 M cells/mL would impact tissue development post-printing. Bright field images after printing and at later time points are shown in Figure 7.5A. The cellular filaments clearly condense into a darker cellular filament, confined by the physical boundaries provided by the alginate filaments. Under PLM, large bands of vivid colour can be clearly visualised after 14 days,

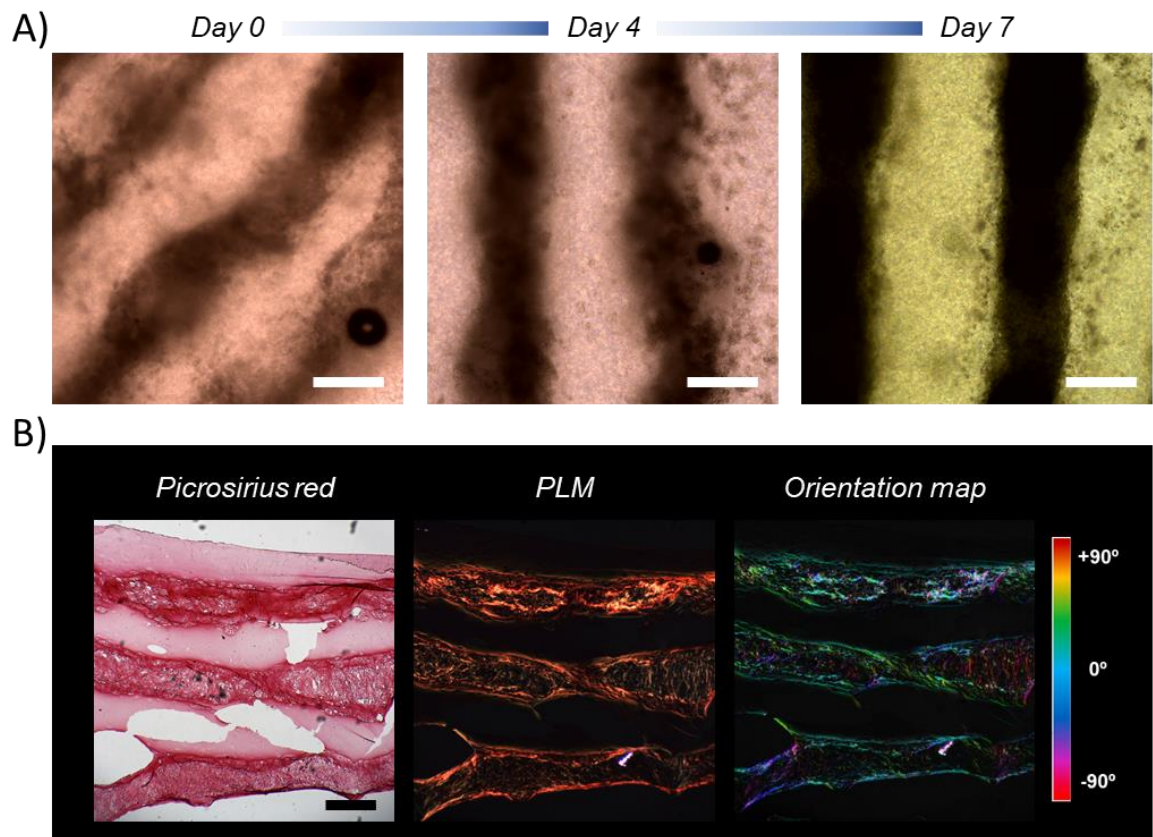


Figure 7.5. A, Phase contrast images of the cells coalescence within the alginate boundaries after the bioprinting step to day 7 in culture. Scale bar is equal to 400 μm . B, Picrosirius red, polarised light (PLM) and colour map imaging of the collagen fibre distributions within the bulk hydrogel. Scale bar is equal to 400 μm .

indicating a degree of alignment (Figure 7.5B). Quantification of the collagen organisation corroborated these observations (Supporting Figure S7.2).

7.4. Discussion

The application of physical boundaries to living cells has previously been used to direct the organisation of the extracellular matrix in engineered tissues. Specifically, polymeric PCL microchambers have been shown to support cellular condensation and direct tissue organisation [7, 221, 244, 245, 345]. This chapter explored the use of printed alginate hydrogels as physical boundaries to direct cellular self-organisation and guide the deposition of the subsequent collagen network. To this end, two different bioinks were used: (1) a fast-degrading bioink and (2) a slow-degrading bioink. These different bioinks have previously been shown to support the in vitro chondrogenic differentiation of MSCs [343]. Cells within the rapidly degrading bioink are able to coalesce and self-organise, while the slow-degrading bioinks functioned as physical boundaries with the potential to direct the spatial organisation of the engineered tissue. By directing cellular self-organisation in vitro by providing appropriate boundary conditions, it may be possible to engineer constructs that not only mimic the anatomical shape, but also the internal microarchitecture of the meniscus tissue.

The encapsulation of cells within the rapidly degrading bioinks allowed them to self-organise within the bulk hydrogel. In Chapter 3 it was demonstrated that the degradation rate of alginate hydrogels could be controlled by oxidising the polymer. The oxidation of alginate above 4% leads to the formation of a very weak hydrogel that dissolves within a very short period (less than a week), making them unsuitable for the current application for which a longer time is required for cells to stabilise the initial 3D printed shape by depositing extracellular matrix components. It was hypothesised that a slow-degrading bioink could act as a supporting material to the rapidly degrading bioinks, thus preventing the cellular structures from collapsing during in vitro culture. Live/dead images show how cells can self-organise within the fast-degrading bioinks, following the

geometry of the external boundary provided by the slow-degrading bioink. This self-organisation, however, resulted in gaps within the printed cellular filaments, which is likely due to the increased cell-mediated contractions in MSC cultures [242, 243].

The use of such hydrogel boundaries can lead to the formation of user-defined neo-tissues composed entirely of cells. In the literature, two are the major concerns when utilising hydrogels for tissue engineering: (1) concerns associated with a negative *in vivo* response (e.g., a foreign body response) to the implanted material, and (2) it may limit the capacity of cells to interact and remodel the engineered tissue [217]. The described approach, therefore, partially mitigates such concerns as the described strategy permits cell interaction, while the programable degradation of the bioinks reduces the amount of foreign material at the end of the *in vitro* culture. By inducing the complete dissolution of the alginate hydrogel using sodium citrate, we could obtain cylindrical neo-tissues composed entirely of cells and their deposited matrix. This approach was used as a proof-of-concept, and future work should develop cell-friendly strategies to dissolve the supporting polymeric matrices by finely controlling the amount of sodium citrate or using supporting bioinks with defined degradation rates [268, 343].

The bioprinted architecture and design were found to influence the alignment of the collagen network laid down by MSCs, but also their biosynthetic activity and the resulting biomechanical properties of the engineered tissues. Following previous work, which has demonstrated that the geometry of the physical boundaries influences the resultant organisation of the bioprinted tissue, we alternated the deposition of non-intersecting filaments of cellular and boundary bioinks, with and without an offset pattern after every other layer. As a control group, we used non-oxidised alginate as the use of oxidised alginate would lead to the complete loss of the construct by the end of the *in vitro* culture period. The amount of sGAG was higher in the control alginate group, which can be linked to different cell microenvironments (e.g., stiffness the cells sense) and the presence of negatively charge aldehyde groups that can interact with the negatively

charged sGAGs [135, 149]. Interestingly, the aligned pattern resulted in higher matrix component accumulation and enhanced mechanical properties. Such a result may be linked to the uncontrolled self-organisation of the cells as seen from the histological images, which indicates the importance of carefully designing the physical boundaries limiting cell self-organisation.

The use of high (60×10^6 cell/mL) cell density bioinks improved the collagen network organisation in comparison to lower (20×10^6 cell/mL) cell densities. Such an effect has previously been linked to the low transport capacity of the collagen fibril precursor molecules within alginate-based hydrogel matrices [376]. The present work shows how the fibrillar collagen was located within the pericellular regions in the control alginate group. Although here it was assumed that the degradation of the rapidly degrading bioink would be unaffected by being confined by another hydrogel, further characterisation is required to effectively demonstrate this, as this process is controlled by the outward flux of ions into the surrounding environment [134]. While high and low cell densities were cultured for different amount of times, it is clear that using high cell densities improved collagen fibrillation as seen under PLM.

7.5. Conclusion

In this work, a novel strategy to control tissue organisation within bioprinted hydrogels was described. Here, a rapidly degrading alginate-based bioink was used to spatially pattern the cells, which were physically confined by a slow-degrading alginate bioink acting as a boundary to the cells. Such a strategy overcomes issues with maintaining the initial printed shape of the rapidly degrading bioinks while still permitting the cells to self-organise within a bulk hydrogel. By optimising the printed design and the cell density, some limited control of the directionality to the collagen network laid down by the cells is possible. In the context of tissue engineering and bioprinting, these temporal bioinks show significant potential for the *in vitro* biofabrication of spatially organised tissues that can potentially be cleared of supporting exogenous biomaterials, ultimately

resulting in ‘scaffold-free’ bioprinted tissues which can recapitulate the microarchitecture of native tissues.

Chapter 8: Discussion

The objective of this thesis was to use a convergence of biofabrication platforms, in combination with tissue-specific progenitor cells derived from the inner and outer regions of the meniscus, to engineer tissues that recapitulated the regional composition, collagen matrix organisation and mechanical anisotropy of the native meniscus.

The thesis began by investigating the development of bioinks that could act as temporal support structures for tissue engineering and bioprinting applications (Chapter 3). Alginate was the selected biomaterial as it is commonly used in the bioprinting of (fibro)cartilaginous tissues [1, 130, 162, 265, 377]. Although mammalian cells do not produce enzymes capable of degrading the polymer, resulting in large amounts of residual alginate still present 8 weeks after *in vivo* implantation, a number of strategies have been investigated to obtain alginate-based hydrogels with faster degradation profiles [267]. Such strategies include the use of gamma irradiation, oxidation, lower polymer densities, or the use of calcium-chelating compounds such as sodium citrate to induce the controlled or predictable dissolution of the gel [135, 137, 268-270]. Partial oxidation of the polymer was chosen as a modifier since the degradation profile of the hydrogel thus depends on the oxidation percentage, which can be easily controlled by the stoichiometric addition of sodium periodate (NaIO_4). Chapter 3 shows how the combination of partially oxidised and unmodified alginate enabled fine control of hydrogel degradation, while maintaining the initial 3D structure of bioprinted constructs. The rate of degradation was found to influence cell clustering, with partial oxidation (4 and 5%) permitting significant cell-cell interaction after 24 - 48 hours in culture. However, the partial oxidation of the polymer decreased the stability and stiffness of the resulting hydrogels, which physically collapsed during *in vitro* culture, causing changes in the initial shape of the construct and the loss of encapsulated cells. By blending oxidised and unmodified alginate, tight control of the rate of degradation was achieved, while the incorporation of gelatine conferred the

bioinks with rheological properties compatible with extrusion bioprinting [128]. All the different bioinks supported high levels of cell viability and robust chondrogenic differentiation of MSCs. Furthermore, the different alginate blends supported comparable levels of sGAG and collagen deposition, demonstrating that the altered biophysical properties of oxidised alginate-containing inks did not negatively impact their capacity to support robust new tissue formation.

As mentioned previously, alginate oxidation also affects the mechanical properties of the resulting hydrogel. It is known that stiffness and viscoelasticity regulate cell behaviour and chondrogenesis, which in native tissues is dictated by the fibrillar architecture of the collagen component [149, 288, 378, 379]. In the present study, only stiffness was measured, with higher oxidised alginate content associated with softer mechanical properties. The ionic crosslinking of alginate allows the polymer to flow under stress, allowing these hydrogels to display varied mechanical behaviours that can be adjusted by altering the molecular weight (e.g., low molecular weights result in fast relaxing hydrogels compared to using high molecular weights) [380]. Furthermore, hydrogel degradation enhances matrix stress relaxation behaviours [381]. Future work would benefit from the development of bioinks with different degradation profiles whilst also controlling for other key factors such as hydrogel stiffness and stress relaxation, something that can be obtained by the combination of high and low molecular weight alginate networks, for example [276]. In summary, in Chapter 3, a strategy was developed to obtain bioinks that can act as temporal support structures for tissue engineering and bioprinting applications.

The next step in this thesis was developing a bioprinting strategy that enables the engineering of spatially organised fibrocartilage tissue. In Chapter 4, inkjet bioprinting was used to deposit a cell-laden bioink (which had been developed in Chapter 3) into polymeric scaffolds fabricated by FDM and MEW. It was found that scaffold architecture can be tailored to direct collagen organisation, supporting the development of tissues with

anisotropic mechanical properties. While existing bioprinting strategies have had some success in recapitulating certain compositional or structural features of the meniscus, engineering tissues with an anisotropic collagen architecture that mimics that of the native tissues remains a challenge [3, 142, 183]. It was previously shown that the introduction of an appropriately designed PCL framework can be used guide cartilage tissue development *in vitro*, resulting in the formation of a stratified biomimetic collagen architecture similar to that seen in articular cartilage [221, 244, 245]. Such studies suggested that 3D (bio)printing could be used to impose physical cues upon cells in a controlled manner, thereby enabling the engineering of tissues with user-defined organisation. Building on those findings, MEW and FDM were employed to manufacture PCL scaffolds with pores of different aspect ratios. These different scaffold pore architectures were found to influence the spatial organisation of the collagen network laid down by the jetted cells, with an increased aspect ratio resulting in the development of highly anisotropic tissues. The high diameter of the PCL fibres produced by FDM compared to MEW resulted in the development of scaffolds that were dramatically stiffer in compression (5 to 10 MPa) compared to native tissue (0.09 to 0.16 MPa) [64]. MEW scaffolds were dramatically softer in compression, but significantly increased with time in culture due to matrix deposition by the cells (0.1 to 0.15 MPa). This reinforcement effect within microfibre scaffolds has been described to be dominated by the load-carrying ability of the scaffold interconnections, where the hydrogel or cellular matrix provides resistance against buckling of the scaffold [185, 197]. This new matrix deposition also enhanced the tensile strength of the resulting constructs (reaching 5.44 MPa parallel to the long axis of the scaffold pores, and 0.37 MPa parallel to the short axis), however they were still noticeably lower than that reported for the human meniscus (between 60 and 200 MPa in the circumferential direction [222]). Recapitulating such native tensile values in highly porous scaffolds fabricated with small-diameter biomaterials is not trivial. Tightly packed electrospun fibre bundles have been shown to achieve strengths of over 100 MPa, however, the high packing density can limit cell and

tissue infiltration [382]. Therefore, the application of bioactive treatments to modulate the collagen fibre network could result in enhanced tensile properties without compromising scaffold porosity and tissue infiltration [170, 178, 328]. These different scaffold architectures not only resulted in different biomechanical performances, but also drove distinct cartilage phenotypes, with decreased collagen type X expression with increased aspect ratio. It is well-known that microfibrillar alignment in electrospun scaffolds can modulate the chondrogenic phenotype of MSCs [238]. Together these observations indicate that scaffold architecture can be used to control both the organisation and phenotype of the engineered cartilage tissue, but also the anisotropic behaviour of the resulting construct. The biofabrication strategy described in this chapter could be used to produce implants for a wide range of biomedical applications.

The application of matrix-modulating factors, such as cABC or lysyl oxidase, has previously been shown to increase the tensile properties of engineered (fibro)cartilaginous tissues [170, 177, 178]. Chapter 5 explored the application of enzymatic treatment, specifically cABC, as a means to improve collagen network maturation and the mechanical properties of bioprinted fibrocartilaginous tissues. Collagen fibril formation is known to be impacted by the presence of GAGs [171]. Notably, abnormally high proteoglycan content has been correlated with a greater degree of tissue disorganisation and impaired mechanical properties [383]. *In vitro*, a negative correlation between GAG content and collagen fibre diameter during the maturation of tissue-engineered fibrocartilage has been suggested [172]. This has driven attention to the temporal removal of GAGs to reorganise and yield denser collagenous tissues with improved tensile mechanical properties [174, 339, 384]. Furthermore, the engineered cartilaginous tissues are most responsive to tension stimulation once the hydrophilic GAGs are removed [181]. In initial investigations, in work led by Dr. Ross Burdis, we observed that the application of cABC on tissue development during the self-organisation of multiple microtissues indeed encouraged tissue (re)modelling and the generation of a cartilage graft with biomimetic composition and structural organisation [173]. This

temporal enzymatic treatment not only yielded denser collagen matrices, but significantly enhanced the fusion between adjacent microtissues within the engineered graft. The convergent bioprinting strategy described in Chapter 4 provided a platform to engineer fibrocartilaginous tissues with user-defined collagen network architectures and anisotropic mechanical properties. However, despite significant *in vitro* tissue deposition by MSCs, the tensile strength and stiffness of the resulting constructs were still orders of magnitude below native properties. Therefore, the overarching goal of Chapter 5 was to study the impact of cABC on the composition, structural organisation and biomechanics of the engineered tissues. The use of such catabolic enzymes during tissue development aids in rebalancing the collagen:sGAG ratio within the *in vitro* engineered tissue, which are typically sGAG-rich but collagen-poor. It was found that this temporal depletion of sGAG was associated with an increased collagen fibre diameter without a detrimental effect on cell viability or tissue phenotype. This increase in collagen fibre size was associated with enhanced tensile properties, thus supporting the inclusion of such enzymatic treatments as a means to enhance the functionality of (fibro)cartilaginous grafts.

Chapter 6 of the thesis investigated the potential use of fibronectin selected MPCs from the inner and outer regions of the meniscus for bioprinting applications, culminating in a study where such MPCs were spatially deposited within wedged-shaped, large-volume MEW scaffold. The identification of an adequate cell population for meniscus regeneration is crucial for a satisfactory regenerative outcome. Encouraged by a number of studies in the literature reporting the use of differential adhesion to fibronectin for the isolation of a progenitor cell population from inner and outer regions of the meniscus, this thesis explored their application to engineer fibrocartilaginous tissue phenotypically similar to that within the inner and outer regions of the meniscus [113, 114, 118]. Fibronectin selected MPCs from the inner and outer regions of the meniscus maintained their differentiation capacity with prolonged monolayer expansion, opening their application within advanced biofabrication strategies that require large numbers of cells.

By depositing MPCs within MEW scaffolds with elongated pore shapes (as described in Chapter 4), which functioned as physical boundaries to cell growth and extracellular matrix production, phenotypically distinct fibrocartilaginous tissues mimetic of the native tissue were obtained.

This thesis also explored an iterative MEW printing process to obtain large-volume wedged-shaped scaffolds recapitulating the profile shape of the native meniscus [206]. The large number of cells deposited within the scaffold raised concerns around nutrient availability, which typically hinders biosynthesis and results in non-desirable gradients in matrix deposition within engineered tissues [363]. To overcome such limitations, sacrificial channels were printed within the scaffold to enhance nutrient diffusion within the large-scale constructs. Additionally, this work employed dynamic culture conditions, which has previously been shown to enhance the quality of *in vitro* engineered cartilaginous tissues [245, 369]. After 6 weeks *in vitro*, MPCs generated a more fibrocartilaginous tissue in comparison to MSCs, which yield to a more hyaline-like phenotype rich in collagen type II.

While this bioprinting strategy clearly enabled the biofabrication of fibrocartilage that mimics key features of the native tissue, there are still areas for improvement:

- Porcine cells were used for all the studies in this thesis. Although the pig is a commonly used preclinical large animal model in many fields of medicine, studies are required to replicate these findings using human derived cells [385, 386].
- The inflammatory environment of an osteoarthritic joint can activate degenerative pathways within meniscus cells, which questions their use for tissue engineering environments. It has been suggested that such degenerative shift may be reversible upon TGF- β treatment [111]. This indicates that a well-defined characterisation of different MPC populations is required before their clinical application. This should include subcutaneous *in vivo* model studies (e.g., mouse) to demonstrate the phenotypic stability of tissues derived from MPCs in comparison to the MSCs.

Furthermore, the bigger question is how such cells (or indeed any cell) will respond once implanted back into an inflamed environment. Therefore, halting the inflammatory cascade while simultaneously restoring functionality is of great interest [387].

- The scaffold developed in this thesis mimicked the wedged shape of the native tissue, future work should focus on translating that into semilunar shapes to recapitulate the macroscopic structure of the meniscus. Another consideration for the development of the next generation of MEW scaffolds for meniscus tissue engineering is the printing on non-planar surfaces. The following work was done in collaboration with Dr. Kian Eichholz (Figure 8.1A). MEW prints are generally performed over flat surfaces to avoid instabilities in the electric field, which defines the macroscopic geometry of MEW scaffolds. Although MEW allows for high-resolution printing, maintaining well-defined edges is challenging (Figure 8.1B). Because of that, there has been an increased interest in the use of non-planar surfaces to recapitulate complex anatomical shapes and curvatures (e.g., femoral condyle curvature) [208-210, 388]. An approach like this could therefore better recapitulate the curvature of the wedge-shaped profile of the native meniscus.

In the initial chapters of this thesis, it has been shown how *in vitro* cells can sense and respond in the presence of printed physical boundaries. These guiding structures were made of PCL, which has shown slow degradability *in vivo* and can undergo plastic deformation upon stress, which could help explain the low clinical translation of such constructs [7, 188]. The aim of Chapter 7 was to translate the concept of physical boundaries to the use of alginate hydrogel structures to act as boundary conditions to direct cellular self-organisation. Previously, the bioprinting of high-density cell

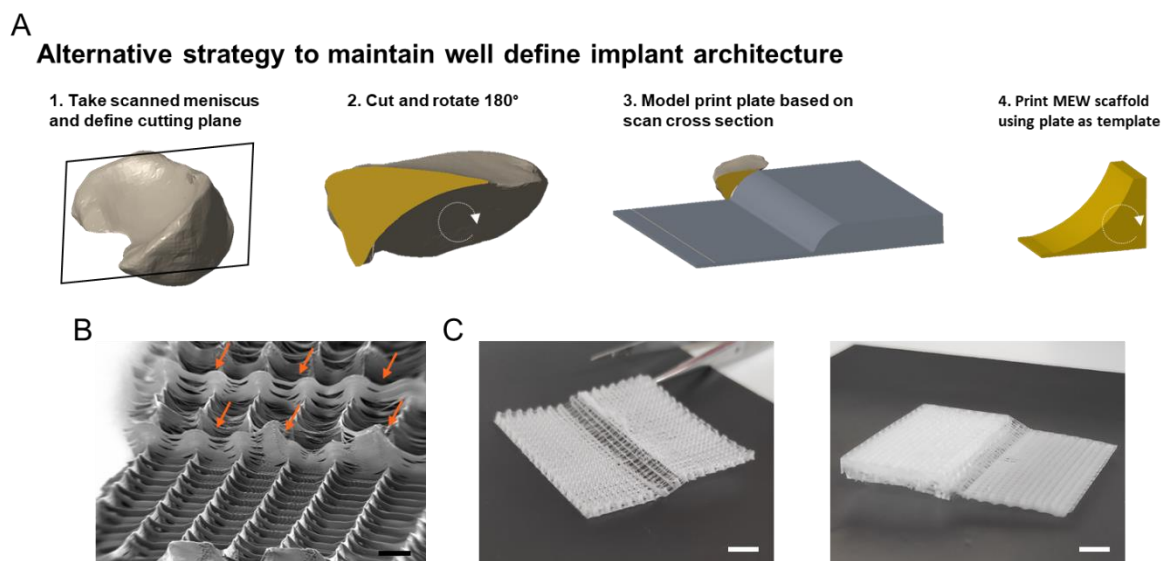


Figure 8.1. Printing on non-planar surfaces of anatomically relevant meniscus-shaped MEW scaffolds
– **Work done in collaboration with Dr. Kian Eichholz.** (A) Schematic representation of the strategy by which curved shaped structures were designed to approach the geometry of a 3D scanned meniscus. (B) Peaks around the scaffold edges indicated by the orange arrows. Scale bar is equal to 1 mm. (C) Successful prints of up to 2 mm in height slopes. Scale bar is equal to 4 mm.

suspensions within hydrogel matrices has been shown to support the self-organisation process of the cells following the printed geometry [246]. Furthermore, individual tissues *in vivo* never develop in isolation but are rather surrounded by other tissues and organs, which mechanically confine, impinge upon, or pull on them [389]. Here, the bioinks developed in Chapter 3 were combined; specifically, a rapidly degrading cellular bioink and acellular unmodified alginate bioink. The rapidly degrading ink was used to spatially deposit the cells, while the unmodified ink acted as the physical boundary providing well-defined geometrical cues. As seen in rapidly degrading bulk hydrogels (single hydrogel component), cells were able to self-organise within the bioprinted constructs following the geometry of the unmodified alginate boundary. Interestingly, the use of high (60×10^6 cell/mL) cell densities improved the organisation of the resulting tissue. This could be due, at least in part, to the lower oxygen levels in high cell density cultures, which is a known factor regulating chondrogenesis and stem cell fate [390-392]. Earlier work has also linked the poor collagen fibril formation within alginate hydrogels to the limited

diffusion of collagen fibril precursor molecules to the pericellular regions, similar to what is seen in the present work within the alginate-only group [376]. Further work is required to characterise this system (e.g., better define the boundary spacing, or define the effect of the boundary stiffness/density). Although very preliminary work, this study demonstrates that hydrogel boundaries can be applied to direct tissue organisation, which combined with the spatial patterning capacity of 3D bioprinting can potentially be used to recapitulate native organisational features. Further bioprinting resolution (lower printing fibre diameter), if required, can be achieved by means of suspended bioprinting in which the bioprinting step is performed within a suspension medium supporting the deposited 3D material [393]. A potential method is the use of freeform reversible embedding of suspended hydrogels (FRESH), which is a slurry composed of gelatine microparticles that display a pronounced shear-thinning behaviour (Figure 8.2A) [394]. This approach drastically improves the resolution achieved through extrusion printing without the need of adding viscosity enhancers (e.g., gelatine) into the bioink (Figure 8.2B).

Together, this thesis describes novel methodology to engineer organised tissues for meniscus regeneration, providing insights into the appropriate combinations of

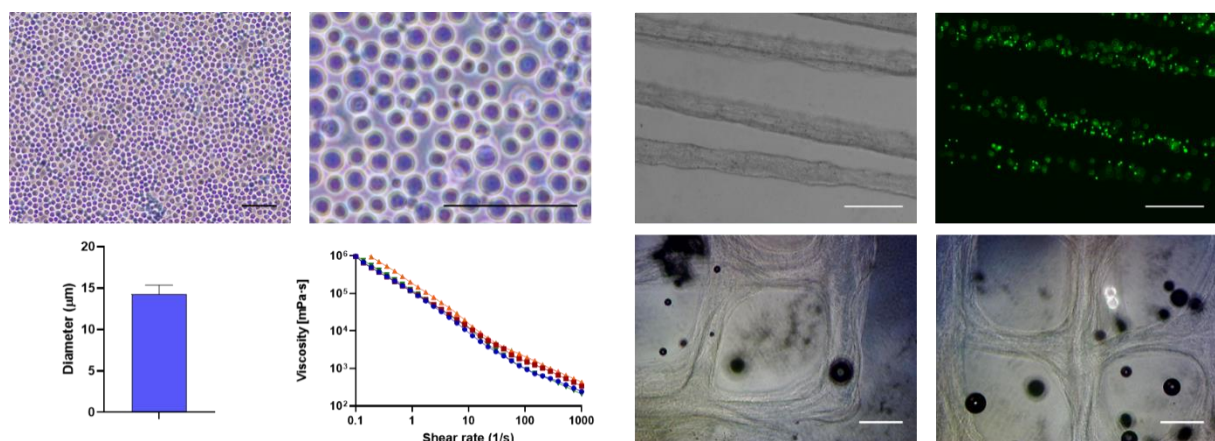


Figure 8.2. High-resolution 3D bioprinting using FRESH. (A) Representative images of the gelatine microparticles. Scale bar is equal to 100 µm. Microparticles size quantification. Rheological properties of the FRESH slurry (n = 4). (B) Single filament printing of alginate-only bioinks. Scale bar is equal to 500 µm.

biophysical, biochemical, and biological factors that can be used to promote meniscus regeneration.

8.1. Limitations of experimental work

Repeatability and reproducibility of experiments are crucial aspects of scientific research that contribute to the robustness and reliability of the obtained results [395, 396]. Repeatability refers to the ability to replicate an experiment by following the same procedures and conditions, ideally producing consistent outcomes. Reproducibility, on the other hand, involves conducting an experiment using different resources, equipment, or researchers while still obtaining similar results. Both repeatability and reproducibility are essential for validating scientific findings and ensuring the accuracy of the reported data.

The current work described experiments performed with only one biological replicate, one of the caveats of interpreting experimental data with only one biological replicate is the limited ability to assess the variability and account for potential outliers or chance occurrences. Biological systems inherently exhibit natural variability, and relying on a single replicate makes it difficult to determine if the observed results are truly representative of the general trends or if they are influenced by random fluctuations. Additionally, with a single replicate, it is challenging to assess the statistical significance of the observed effects and draw robust conclusions.

To address these limitations, it would have been highly recommended to perform experiments with multiple biological replicates. By including multiple replicates, it would have been possible to account for natural variability, assess the consistency of the results, and improve the statistical power of the different analyses. Replicates help to identify and mitigate the impact of outliers or anomalous observations, thereby providing a more accurate representation of the experimental outcomes.

Performing a power analysis is a valuable tool in experimental design. It involves estimating the appropriate sample size needed to achieve sufficient statistical power to detect meaningful effects or differences between groups. Power analysis takes into account factors such as the desired level of statistical significance, effect size, variability, and the chosen statistical test. By conducting a power analysis, researchers can optimise their experimental design, ensuring that they have an adequate sample size to detect meaningful effects while minimising the risk of false-negative results.

In summary, repeatability and reproducibility are fundamental for establishing the credibility of scientific experiments. Conducting experiments with multiple biological replicates enhances the reliability and generalisability of the findings. Moreover, performing a power analysis during experimental design helps determine the optimal sample size, improving the statistical power and accuracy of the results. By prioritising repeatability, reproducibility, and thoughtful experimental design, it is possible to enhance the reliability and validity of scientific investigations.

8.2. Challenges and future perspectives

While the presented research offers promising advancements in the field of meniscus tissue engineering using 3D bioprinting, there are still several challenges and areas for further investigation. Addressing these challenges and exploring future perspectives will be crucial for the successful clinical translation of this technology. Some important considerations include:

Size matching is a critical factor when investigating and developing meniscal grafts. The exceptional conformity of the menisci allows for the dispersion of contact forces over a significantly wider area, effectively minimising the contact pressure that can build up on the articular cartilage [397]. The size and shape of the menisci are, therefore, important determinants of the contact pressure distribution and good clinical outcomes. Insufficiently sized grafts can lead to excessive loads due to inadequate congruity with the femoral condyle, while excessively large grafts run the risk of losing

their functionality and being prone to extrusion from the joint compartment [398]. Even a small mismatch of a few millimetres can lead to inferior biomechanical outcomes and increased degenerative changes [399]. Consequently, it is vital to strive for sizing that closely resembles the dimensions of the native meniscus. Currently in use methods for meniscus sizing based on “2D” MRI measurements still provide errors over 5% and considering that a 10% graft mismatch can cause relevant articular problems indicates the need for better sizing methods [400]. These circumstances have led to the development of alternative approaches, such the formation of 3D models from an MRI [398, 401]. The advancements on the meniscus sizing will definitely improve the outcome of meniscus allografts by finding the best fit, but also the detailed 3D reconstruction will definitely have an impact to the biofabrication of personalised implants.

Cell-based therapies for meniscus regeneration have undergone several advancements, utilising different cell sources [38, 402]. However, limitations in terms of patient burden and costly cell-expansion phase have steered meniscus repair and regeneration towards single-stage procedures and off-the-shelf cellular or biomaterial-based products [403]. Due to the low cell number in native meniscus, native cells are required to be culture-expanded to obtain a sufficient number for treatment. Therefore, single-stage strategy would not be feasible if autologous cells were to be used. Investigation on the allogenic use of meniscus-derived cells (fibrochondrocytes and MPCs) will be valuable, as such investigations can greatly improve the potential of application. Alternatively, a large number of MSCs can be harvested from various sources including bone marrow, adipose tissue and infrapatellar fat pad [403]. For example, the “Cell Bandage” consists of autologous bone marrow MSCs seeded onto a collagen matrix, which were harvested from the iliac crest two weeks before the surgery [39]. As a true one-stage strategy requires only one surgical intervention, additional biopsies apart from the surgery of any kind to isolate meniscus-derived cells or MSCs should be avoided. This suggests that cells should either be isolated during the time frame of one surgery or allogeneic cells should be used [404]. Although as of yet no preclinical or

clinical studies are ongoing which explore the use of induced pluripotent stem cell (iPSCs) for meniscus repair, a GMP-grade iPSC cell line might become the basis for such a product in the future, providing that cell fate can be controlled. These unlimited self-renewable cells obtained from somatic cell reprogramming can theoretically be used to create patient specific fibrochondrocytes [405-407]. However, this technology comes with a number of challenges including tumorigenesis risk, and low levels in the efficiency to differentiate towards several lineages. Furthermore, studies have suggested that abnormal gene expression and mitochondrial mutations promote the immunogenicity of autologous iPSCs [408]. Despite the promising results of iPSCs to be directed towards mesenchymal lineages including chondrocytes and tenocytes technical challenges are yet to be overcome before realising into a therapeutic option. Finally, it will be important to consider an inflammatory environment, especially with the presence of inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, which can inhibit tissue regeneration, when developing cell-based therapies [91, 406].

Implants for meniscus repair should relieve symptoms after meniscectomy, provide a functional improvement, and in the long term prevent the development of osteoarthritis. Regarding a meniscal substitute, two main streams can be observed: first, the construction of permanent prosthesis that mimics the biomechanical function of the meniscus, and secondly to produce a scaffold which allows tissue ingrowth and is biodegradable, encouraging the body to replace its lost tissue [36]. Clinically in use grafts follow the premise that replacing the resected tissue with a supportive biomaterial will improve partial meniscectomy outcome, however, it is only assumed that the long-term outcome will lead to better knee function and protect the articular cartilage from further damage. The observation that the implant is biomechanically stable does not automatically imply that it put forth adequate biomechanical properties within the joint. However, insufficient mechanical characteristics, insufficient tribologic properties, and wrong implant size lead to damage to the articular cartilage comparable to that of meniscectomy. Requirements that were described by Rongen et al, that a meniscal

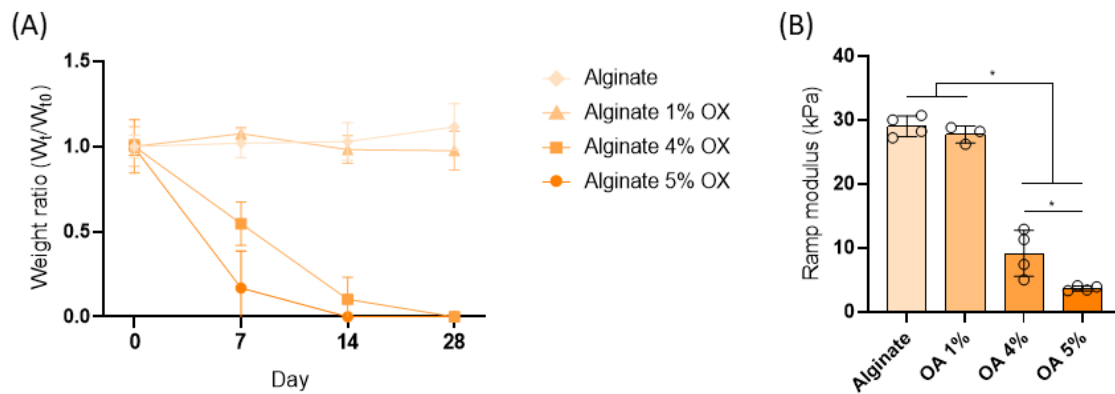
substitute must possess [36]. Design criteria for a biomaterial intended for meniscus repair should consider the target population, overall low cellularity, the dense collagenous matrix, the poor vascularity, and the inflammatory environment present as all these contribute to the failure of meniscus healing and regeneration. Based on this understanding, novel biomaterials may help to address such limitations. Another important consideration when developing biodegradable implants is that *in vivo* biodegradation has to be adjusted to the balance of tissue healing (e.g., by controlling scaffold porosity). Furthermore, local microenvironment including the biological, physical and inflammatory environment necessitate of adjustment for the translation of *in vitro* strategies. Tracking methods that allow the study of the *in vivo* degradation without the need of sacrificing animals can definitely improve the translational outcomes [409, 410]. Overall, the required level of complexity of an effective tissue engineered biomaterial is still a matter of debate, and it is greatly dependent on the intended clinical functionality.

8.3. Conclusions

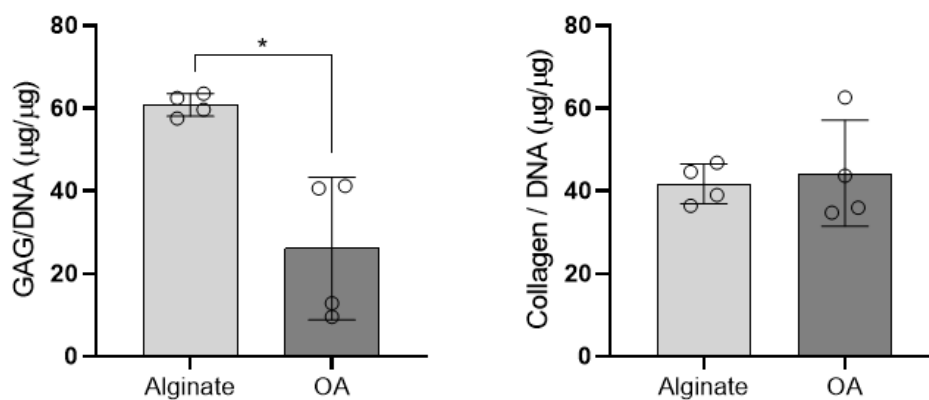
- A toolbox of alginate-based bioinks which can act as temporal support structures for tissue engineering and bioprinting strategies was developed.
- A convergent bioprinting strategy that enables the engineering of soft tissues with user-defined collagen network architectures and anisotropic mechanical properties was developed.
- It has been shown how temporal depletion of sGAGs through enzymatic treatment with cABC modulates matrix composition and enhances the collagen maturation within bioprinted tissues.
- Zone-specific MPCs are a promising cell source for meniscus tissue engineering, maintaining distinct regional phenotypes following printing, including altered ratios of collagen type I to collagen type II expression, and differential expression of regional markers such as TNMD.

- This study demonstrates the feasibility of creating a large-volume wedge-shaped meniscus scaffold with MEW.
- In this thesis, a novel strategy to control tissue organisation within bioprinted hydrogels has been described by spatially patterning bioinks with different degradation rates.

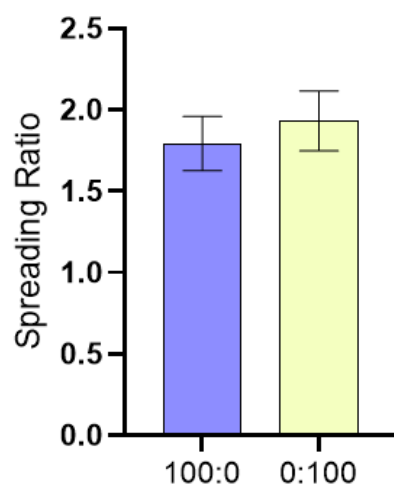
Supporting information



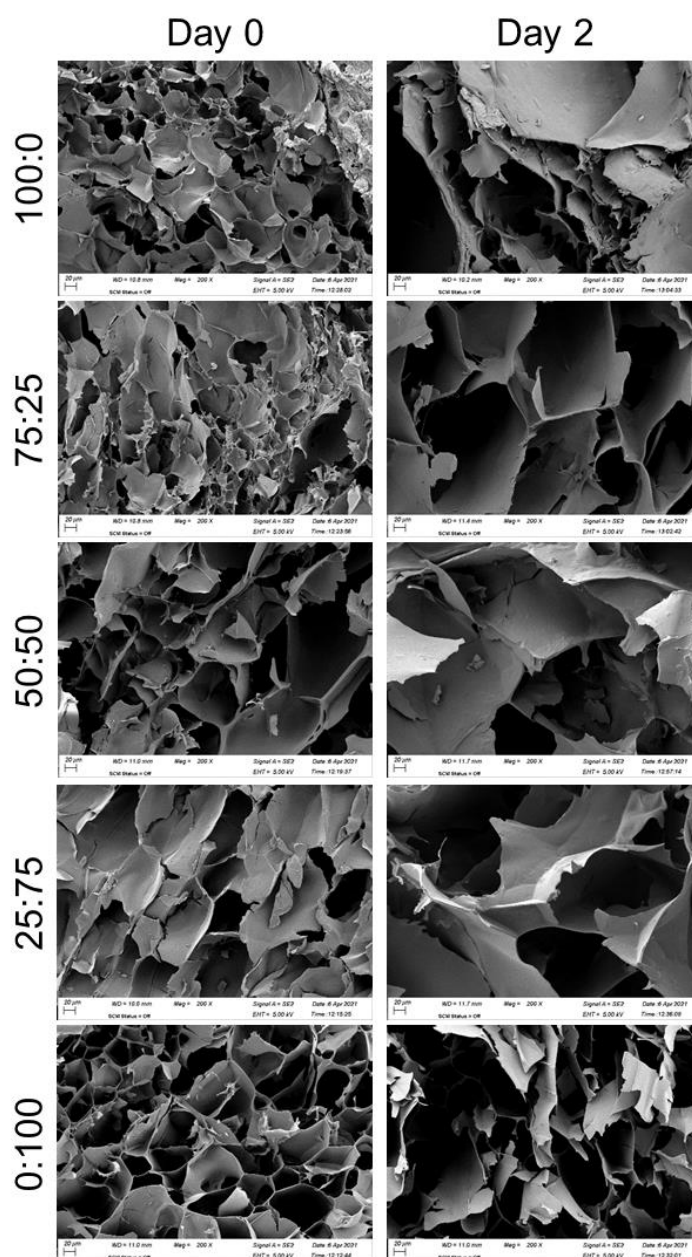
Supporting Figure S3.1. (A) Dry weight of partially oxidised alginate hydrogels at different time points. (B) Ramp modulus of partially oxidised alginate hydrogels. All error bars denote standard deviation, significance was considered $*p \leq 0.05$, $n = 4$.



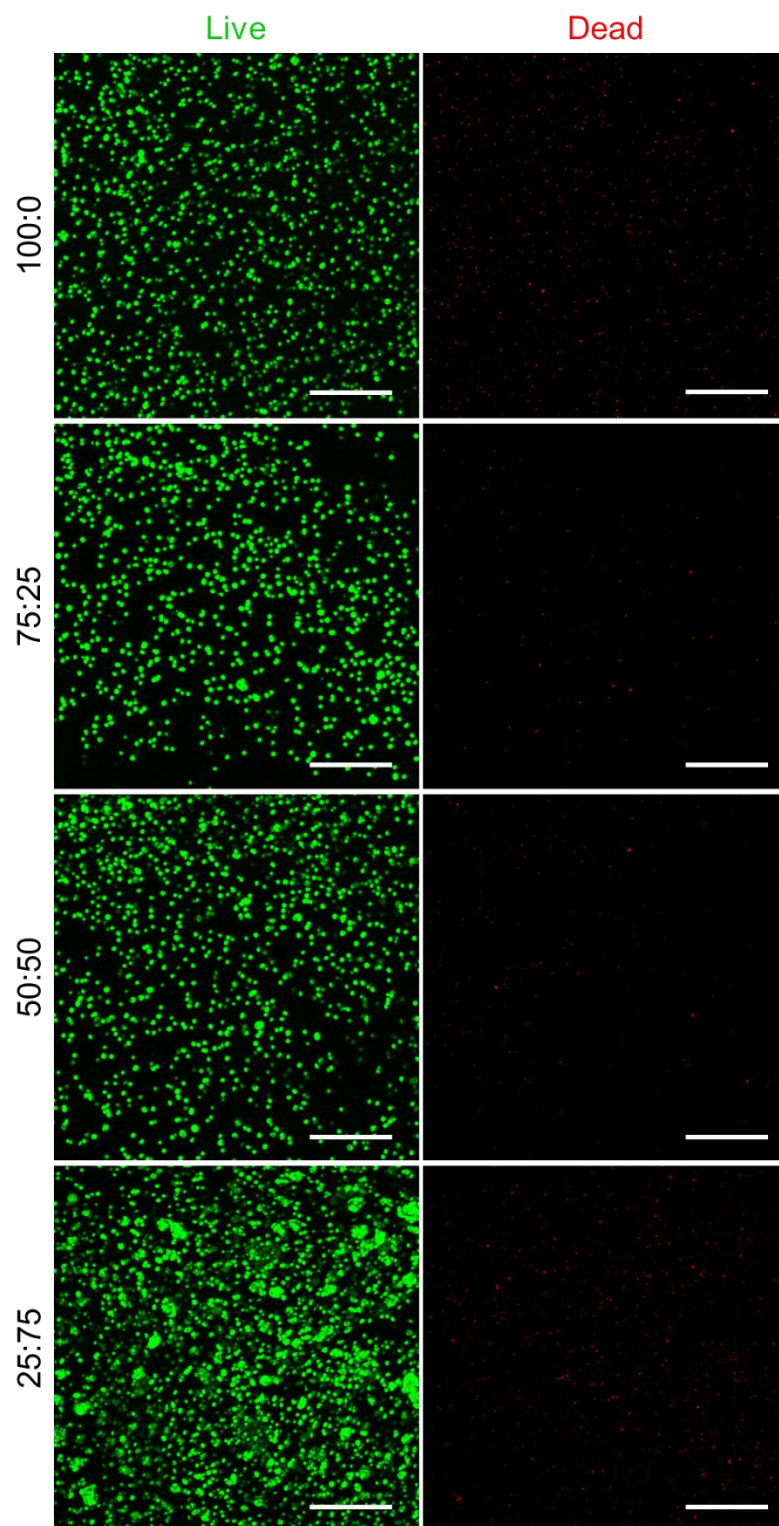
Supporting Figure S3.2. Quantification of sGAG and collagen deposition in alginate and oxidised alginate gels following 28 days of *in vitro* culture. All error bars denote standard deviation, significance was considered $*p \leq 0.05$, $n = 4$.



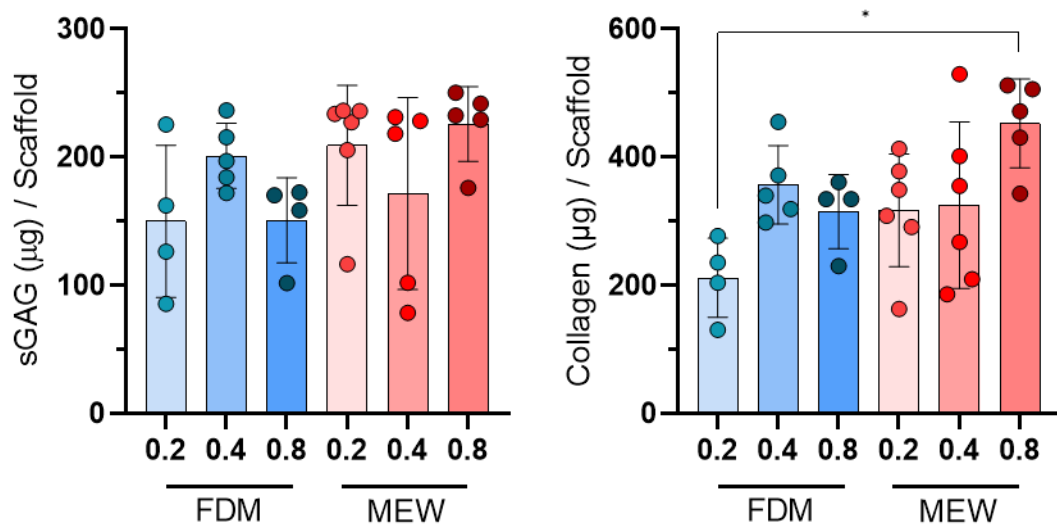
Supporting Figure S3.3. Post printing spreading ratio (width of the filament divided by needle diameter).
All error bars denote standard deviation.



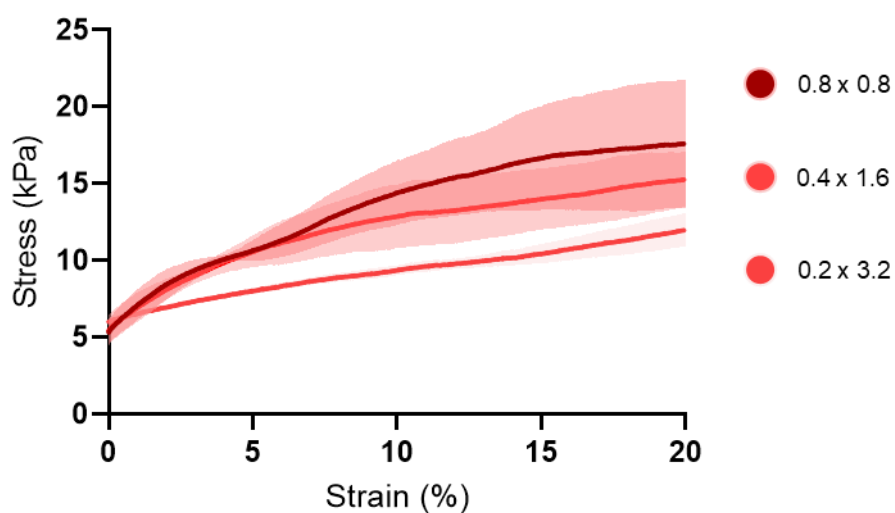
Supporting Figure S3.4. SEM images at Day 0 and Day 2 revealing the increased porosity after the gelatine removal. The scale bars are equal to 20 μm .



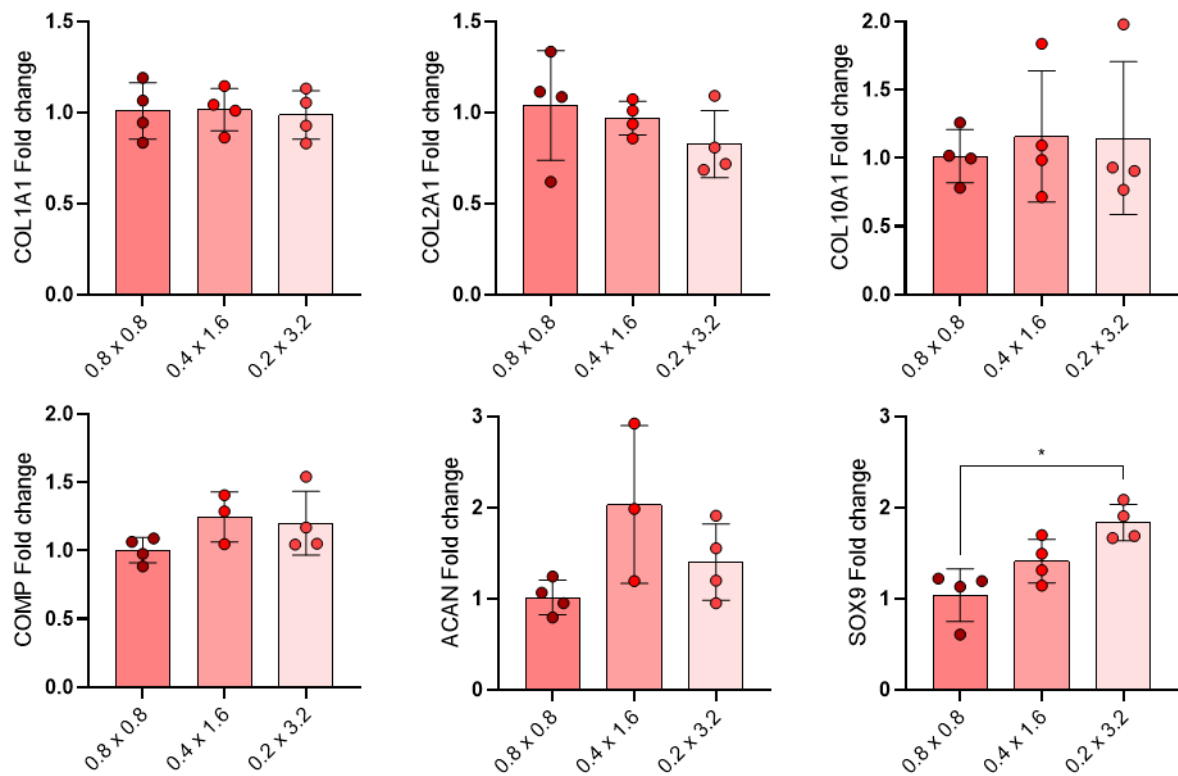
Supporting Figure S3.5. Representative images of the live/dead staining at day 7. Green and red indicate live and dead cells, respectively. Scale bars are equal to 250 μm .



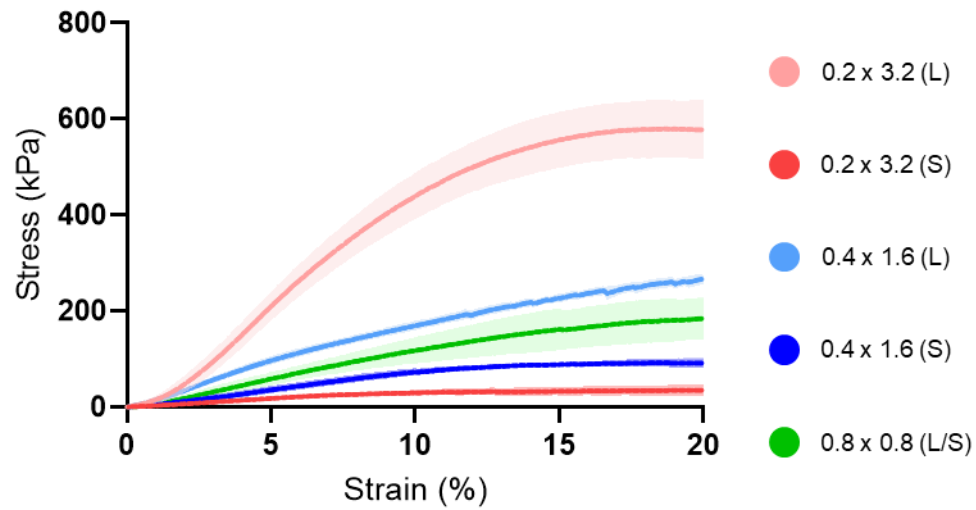
Supporting Figure S4.2. Biochemical analysis following 5 weeks of *in vitro* culture. All error bars denote standard deviation, significance was considered * $p \leq 0.05$, $n \geq 4$.



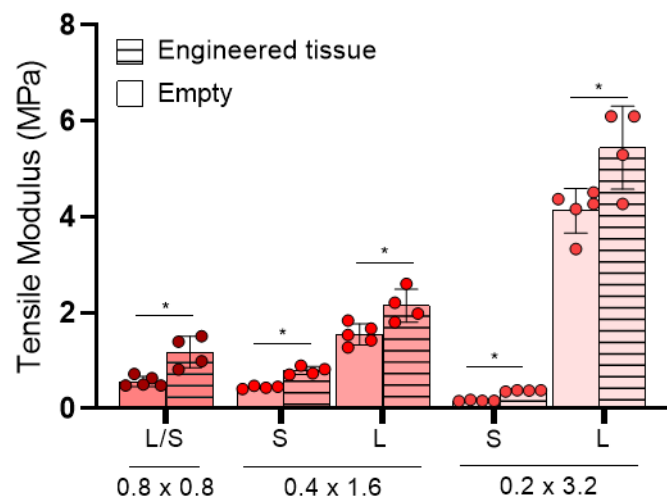
Supporting Figure S4.3. Stress-strain plots of the compressive tests. Dark data points represent the mean, and the lighter area shows the standard deviation ($n=4$).



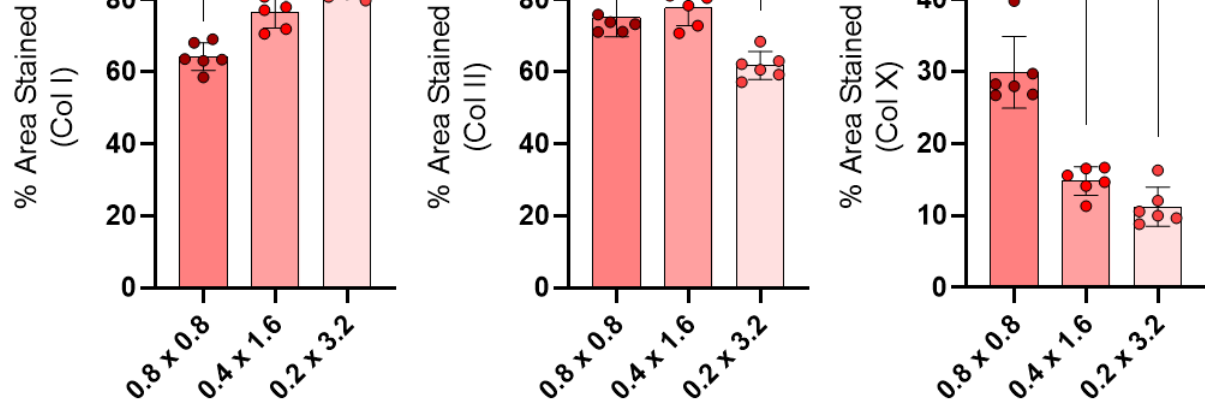
Supporting Figure S4.4. Gene expression at week 4. Collagen type I (COL1A1), II (COL2A1), X (COL10A1), aggrecan (ACAN), SRY-Box 9 (SOX9) and cartilage oligomeric matrix protein (COMP). Data is reported as fold-change relative to the 0.8 x 0.8 mm group. All error bars denote standard deviation, significance was considered $*p \leq 0.05$.



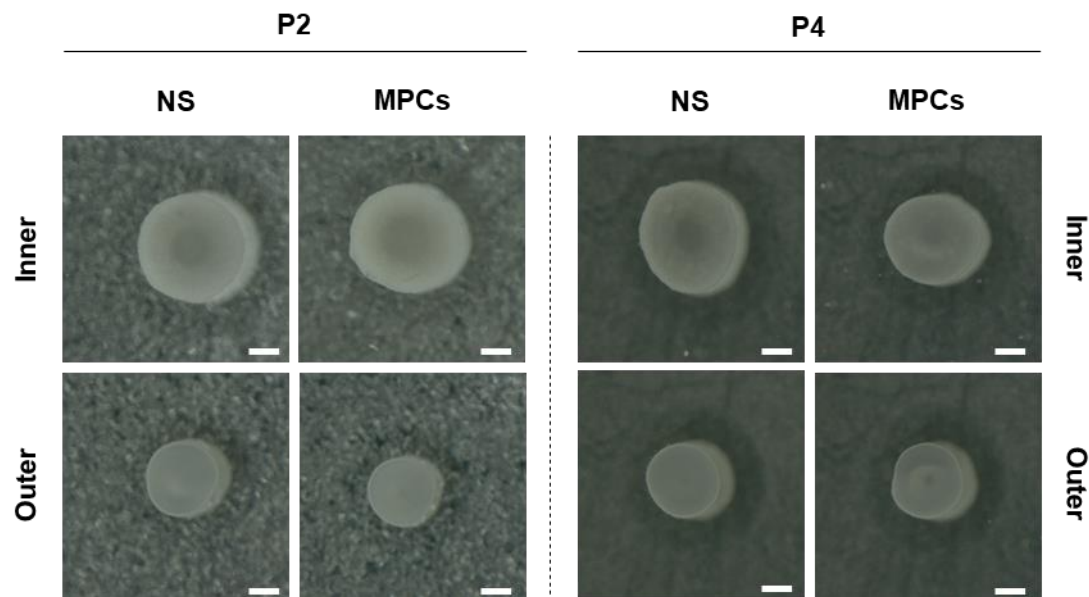
Supporting Figure S4.6. Stress-strain plots of the tensile tests. Dark data points represent the mean, and the lighter area shows the standard deviation (n=4).



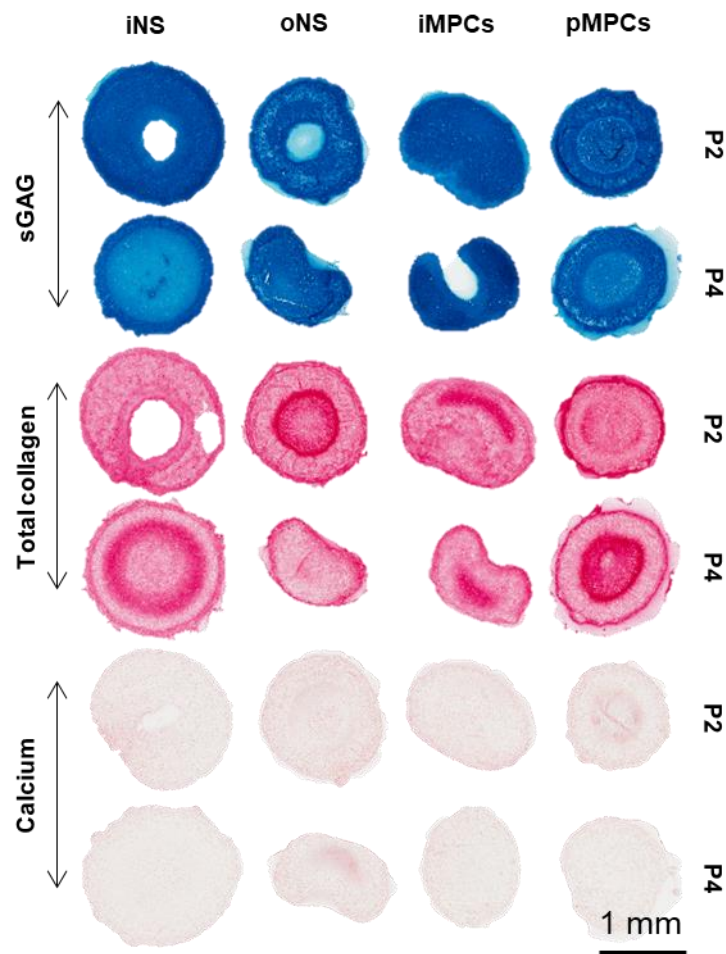
Supporting Figure S4.5. Tensile properties of the engineered tissues compared to empty scaffolds. All error bars denote standard deviation, significance was considered $*p \leq 0.05$.



Supporting Figure S4.7. Percentage of the area stained positive for collagen type I, II and X. All error bars denote standard deviation, significance was considered $*p \leq 0.05$.

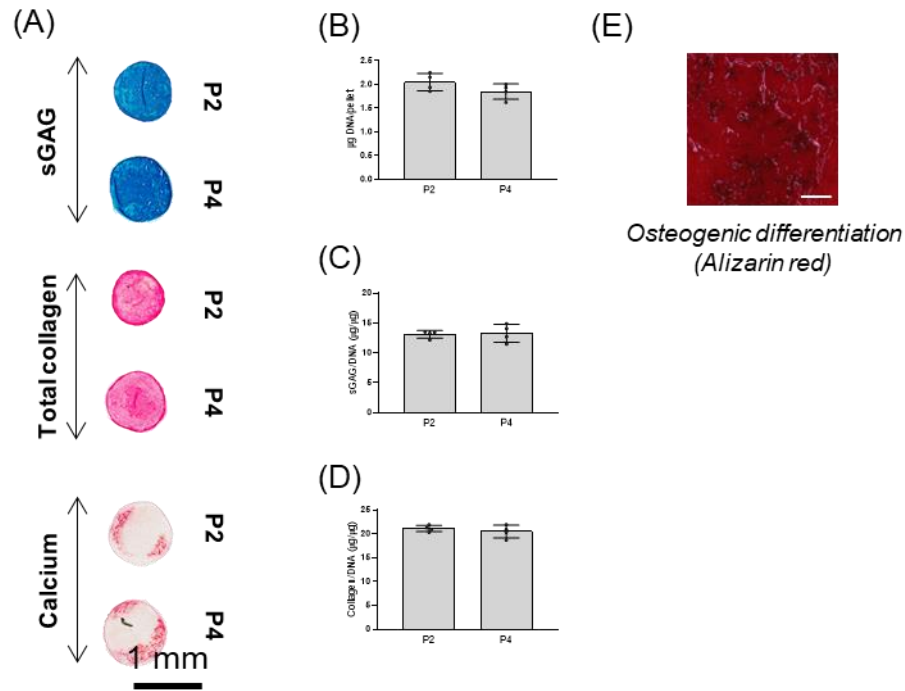


Supporting Figure S6.1. Bright field images of the different meniscus-derived cell populations (each pellet contains 250,000 cells) at passages 2 and 4 of culture. Scale bar is equal to 500 μm .



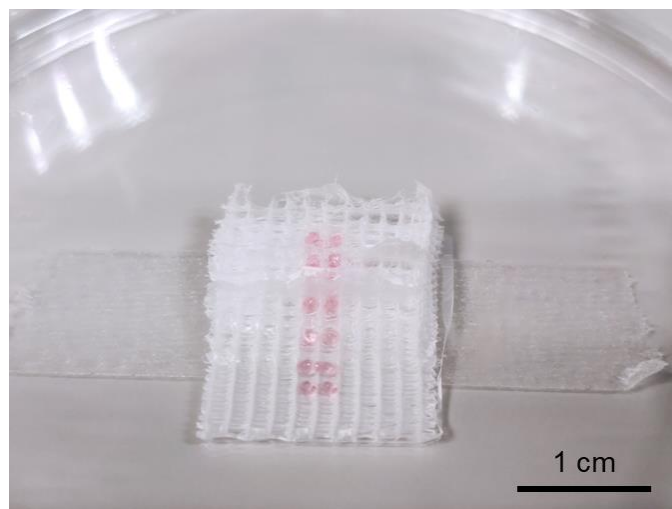
Supporting Figure S6.2. Histological evaluation after 4 weeks in chondrogenic media of the different meniscus-derived cell populations. Alcian blue staining (sGAG), picosirius red staining (total collagen) and alizarin red staining (calcium). Scale bar is equal to 1 mm.

MSCs

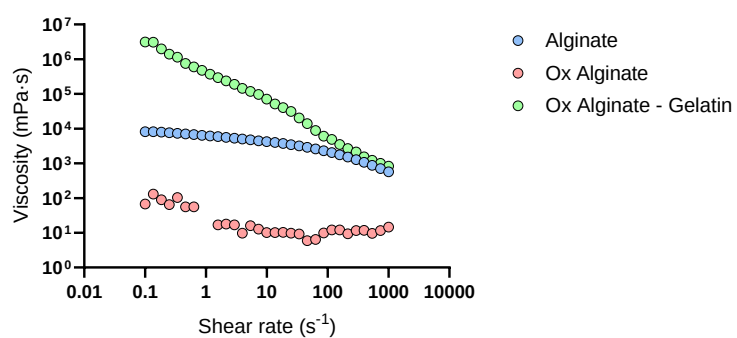


Supporting Figure S6.3. Chondrogenic and osteogenic potential of porcine derived bone marrow MSCs.

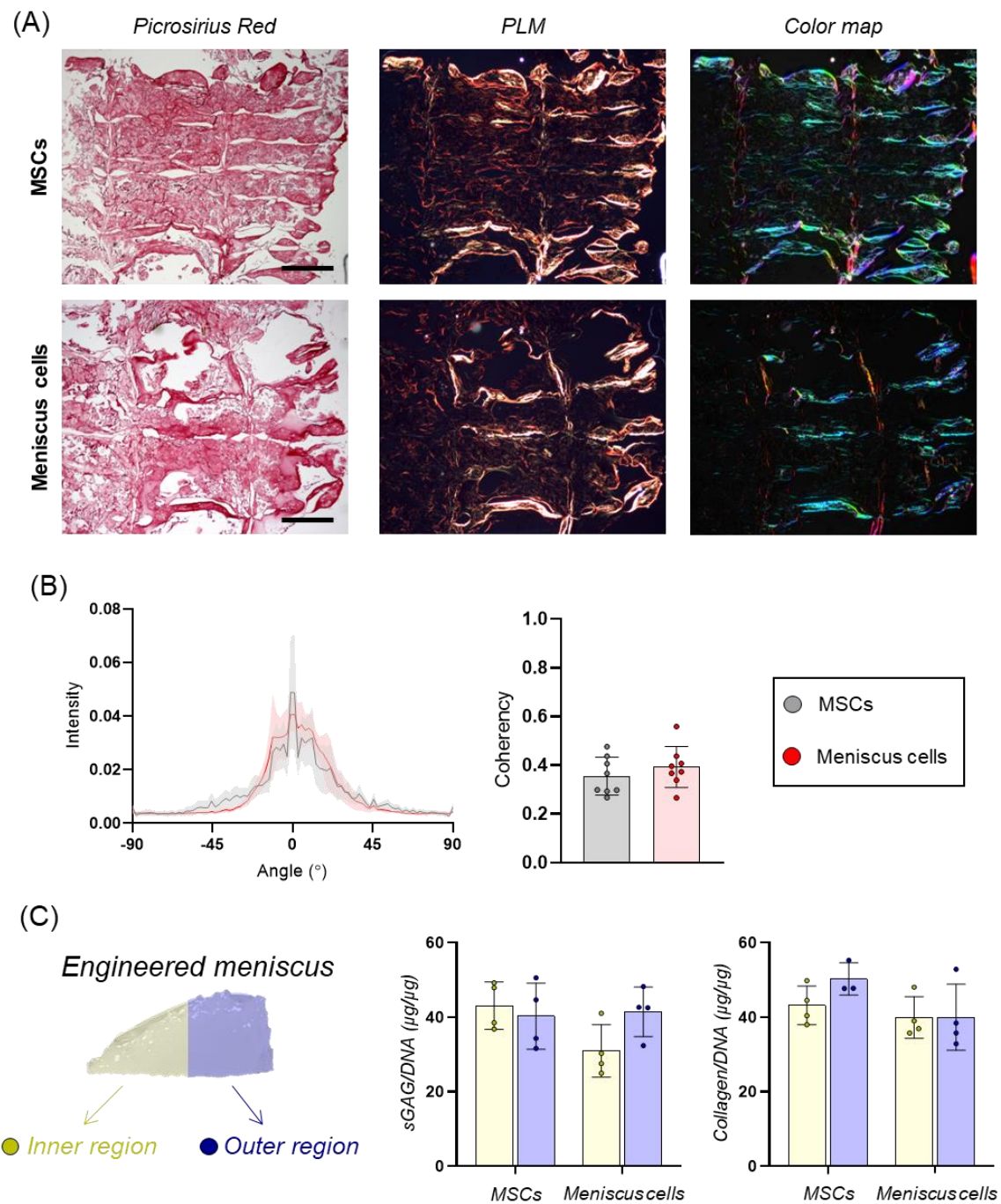
(A) Histological evaluation after 4 weeks in chondrogenic media. Alcian blue staining (sGAG), picrosirius red staining (total collagen) and alizarin red staining (calcium). Scale bar is equal to 1 mm. Biochemical analysis at day 28 for the different cell populations, (B) DNA content, (C) sGAG content and (D) collagen content. (E) Alizarin red staining following 3 weeks of culture with osteogenic media. All error bars denote standard deviation, n = 4.



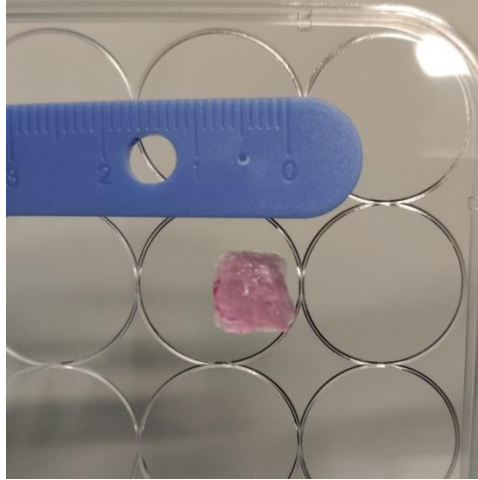
Supporting Figure S6.4. Application of gelatine in the wedge-shaped MEW scaffold as sacrificial hydrogel to create nutrients diffusion pores after washing out. Scale bar is equal to 1 cm.



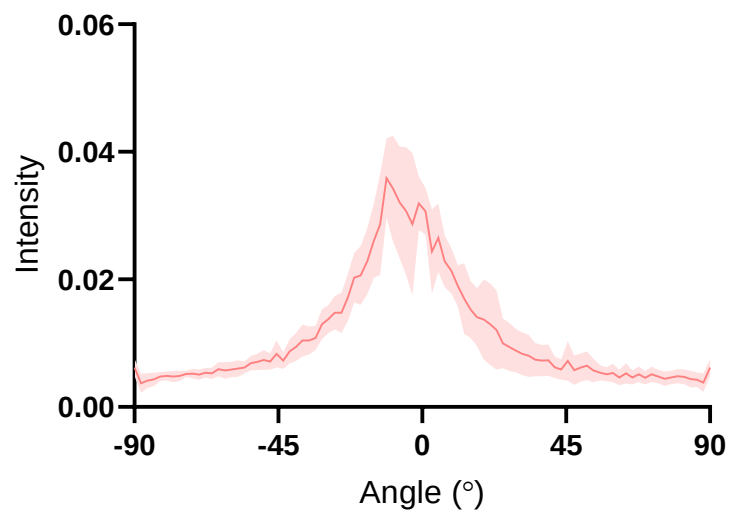
Supporting Figure S6.5. Viscosity at 16 °C of unmodified, oxidised alginate, and the oxidised alginate-gelatine bioink in the presence of a shear rate in the range of 0.1 to 1000 s⁻¹.



Supporting Figure S6.6. (A) Collagen fibre orientations within the wedge-shaped scaffold following 6 weeks of culture. Scale bars are equal to 800 μm . (B) Quantification of the fibre orientation. Quantification of the fibre coherency, where values approaching to 1 indicating that fibres are aligned towards a preferential direction. All error bars denote standard deviation. (C) Biochemical analysis within the inner and outer region of the engineered construct. All error bars denote standard deviation.



Supporting Figure S7.1. Alginate hydrogel before degradation (Figure 7.2, top-right) using sodium citrate.



Supporting Figure S7.2. Quantification of the relative number of fibres oriented in a specific direction.

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