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AN EMBEDDED BIOPRINTING PLATFORM TO GUIDE THE
FUSION, PHENOTYPE AND REMODELLING OF
MICROTISSUES TO ENGINEER ANISOTROPIC
MUSCULOSKELETAL TISSUE

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Doctor of Philosophy

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university, and it is entirely my own work. I confirm that the work has been completed in full compliance with relevant university policies, including the Academic Integrity policy, the Trinity Policy on Good Research Practice, and the guidance on AI and GenAI in Teaching, Learning, Assessment and Research.

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Francesca Diletta Spagnuolo

Dublin, 2nd March, 2026

A handwritten signature in dark ink, reading 'Francesca Diletta Spagnuolo'. The signature is written in a cursive, flowing style.

Summary

Musculoskeletal tissue injuries are common and difficult to treat due to poor intrinsic healing, often leading to degeneration and osteoarthritis. Current surgical approaches show inconsistent outcomes, driving the need for improved regenerative strategies. Tissue engineering (TE) aims to develop patient-specific constructs that replicate native tissue structure and function. Cellular self-organisation using microtissues (μ Ts) offers a promising route to biomimetic tissue formation through fusion and remodelling. However, controlling μ T spatial organisation remains challenging. This thesis addresses this by developing a bioprinting platform to pattern μ Ts and guide their fusion, differentiation, and extracellular matrix (ECM) organisation toward anisotropic musculoskeletal tissues.

Chapter III investigated the influence of μ T maturation state and density on the engineering of cartilage constructs of clinically relevant size. The μ Ts were formed at different maturation stages (day 2, 4, and 7) to form larger constructs. Less mature (day 2) μ Ts demonstrated faster and more effective fusion, producing an ECM richer in sulphated glycosaminoglycans (sGAG) and collagen, with low calcium deposits. Large-scale constructs formed from 4,000 μ Ts exhibited robust fusion regardless of culture conditions, although dynamic culture did not significantly improve ECM accumulation or mechanical properties. However, this strategy does not recapitulate the native organisation. Integrating μ Ts into 3D bioprinting strategies could enable spatial control over tissue architecture and ECM organisation, improving alignment and mechanical performance.

Chapter IV introduced extrusion-based bioprinting to deposit F-D2 μ T-laden bioinks within xanthan gum (XG)-based support baths. The goal was to define suitable combinations of bioink and support material for the optimal extrusion of μ Ts and post-printing fusion. Gelatin-based bioinks provided superior printability and biological performance compared to methylcellulose. While traditional sacrificial baths enabled printing, xanthan gum (XG)-based systems improved structural stability. A methacrylate XG (XG-MA) bath maintained physical confinement over time, preventing tissue shrinkage and promoting ECM organisation. These findings highlighted the importance of stable boundary conditions in guiding tissue maturation and preserving construct geometry.

Chapter V expanded this approach into a 4D bioprinting platform, where time-dependent changes in tissue organisation were driven by the evolving mechanical properties of the surrounding environment. By modulating support bath stiffness, μ T behaviour and differentiation could be directed under identical biochemical conditions. Softer environments promoted cartilage-like phenotypes, intermediate stiffness supported fibrocartilage formation, and stiffer conditions favoured ligament-like tissues. Mechanical confinement also induced collagen alignment along the printing direction, with increasing stiffness enhancing both fibre organisation and overall tissue anisotropy. This platform demonstrated that support baths can function as active mechanobiological regulators, enabling the engineering of multiple musculoskeletal tissues with controlled architecture and phenotype.

A key limitation identified was incomplete fusion between adjacent printed filaments. Chapter VI addressed this by developing a composite support bath combining a more degradable component. A 1:1 ratio (XG:XG-MA) achieved the optimal balance between structural stability and fusion. While all formulations supported tissue differentiation, the optimised composite bath also preserved anisotropy while improving inter-filament cohesion. Mechanical testing confirmed enhanced load transfer and increased transverse stiffness in these constructs, demonstrating improved functional integration.

Finally, Chapter VII applied the optimised platform to engineer a zonally organised meniscal graft. Growth factor strategies were first evaluated, showing that TGF- β 3 promoted a cartilage-like phenotype, while combined CTGF and TGF- β 3 induced a fibrocartilaginous phenotype characteristic of the outer meniscus. Using laponite-based bioinks for controlled growth factor delivery, large-scale meniscal grafts were fabricated with distinct regional composition, including sGAG- and collagen II-rich inner zones and collagen I-dominant outer zones. This approach achieved a higher degree of zonal organisation than previous methods, while maintaining high cell viability and scalability.

Overall, this thesis establishes a comprehensive framework for μ T-based bioprinting of musculoskeletal tissues. By integrating cellular self-organisation with advanced bioprinting strategies, it demonstrates how mechanical and biochemical cues can be combined to guide tissue formation, ECM organisation, and functional maturation. While further optimisation is required to achieve fully native-like properties, particularly in terms of

collagen content and mechanical performance, the platform provides a strong foundation for future preclinical and clinical applications.

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“Not all those who wander are lost”

Fran

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Publications

First Author publications

F.D. Spagnuolo, Kronemberger G.S., Kelly D.J., Bioprinting of Microtissues Within Mechanically Tunable Support Baths to Engineer Anisotropic Musculoskeletal Tissues, *Advanced Science*, e09313., 2026

F.D. Spagnuolo, G.S. Kronemberger, K.J. Storey, D.J. Kelly, The maturation state and density of human cartilage μ Ts influence their fusion and development into scaled-up grafts, *Acta Biomaterialia*, 194, 109-121, 2025.

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McCarthy C.M, McKevitt K. L., **F.D. Spagnuolo**, Mahon O. R., Leahy F.C., Moloney M. A., Kavanagh E. G. Walsh M. T., Mulvihill J. E., Varicose Veins for Peripheral Bypass Surgery, a Micromechanical and Structural Comparison with Great Saphenous Vein, *Under Review*

G.S. Kronemberger, K. Chattahy, **F.D. Spagnuolo**, A.S. Karam, D.J. Kelly, Bioassembly of Region-Specific Fibrocartilage μ Ts to Engineer Zonally Defined Meniscal Grafts, *Advanced Healthcare Materials*, e02208, 2025.

G.S. Kronemberger, **F.D. Spagnuolo**, A.S. Karam, K. Chattahy, K.J. Storey, D.J. Kelly, Growth Factor Stimulation Regimes to Support the Development and Fusion of Cartilage μ Ts, *Tissue Engineering Part C: Methods* 31 (1), 36-48, 2025.

G.S. Kronemberger, **F.D. Spagnuolo**, A.S. Karam, K. Chattahy, K.J. Storey, D.J. Kelly, Rapidly degrading hydrogels to support biofabrication and 3D bioprinting using cartilage μ Ts, *ACS Biomaterials Science & Engineering* 10 (10), 6441-6450, 2024.

Conference Abstracts

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F.D. Spagnuolo, G.S. Kronemberger, D.J. Kelly, 3D bioprinting of high-density microtissue-laden bioinks for cartilage tissue engineering applications, 1st Alpine Winter School of Biofabrication, Radstadt, Austria, 2024. *Poster Presentation*.

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Chapter I. Introduction

1.1 Musculoskeletal Tissue Engineering

The musculoskeletal system, comprising bones, cartilage, ligaments, tendons, and muscles, is fundamental to human locomotion, support, and protection [1]. Injuries or degenerative diseases affecting these tissues - often resulting from trauma, ageing, congenital defects, or inflammatory conditions - pose a significant global health burden, leading to pain, disability and reduced quality of life for millions [2, 3]. While the human body possesses remarkable self-healing capacities for minor injuries, large-scale defects or chronic conditions frequently overwhelm these intrinsic repair mechanisms, necessitating clinical intervention. Musculoskeletal tissue engineering (TE) has emerged as a transformative interdisciplinary field, integrating principles from engineering, biology and medicine to develop innovative strategies for repairing, replacing or regenerating damaged musculoskeletal tissues [4-8]. The ultimate goal of TE is to restore both the structural integrity and the complex biomechanical functions of these tissues.

For centuries, medical professionals have employed various strategies to address musculoskeletal defects, often relying on surgical reconstruction and repair, such as allografts, autografts or synthetic implants and/or prostheses [9-11]. These methods, while effective for many conditions, frequently face limitations that prevent complete functional restoration [12]. Engineering replacement tissues that can fully restore tissue function is crucial for the successful regeneration of the musculoskeletal system [13, 14].

The field of musculoskeletal TE and regenerative medicine aims to overcome the limitations of conventional treatments by combining cells, biomaterials and regulatory signals to develop advanced biomimetic constructs [15]. Living cells—often stem cells such as bone marrow-derived mesenchymal/stromal stem cells (MSCs) or induced pluripotent stem cells (iPSCs)—act as the regenerative units capable of forming new tissues. These cells are usually supported by biomaterials (e.g. scaffolds), which serve as temporary or permanent structural templates designed to replicate the native extracellular matrix (ECM). Scaffolds, for instance, ideally provide the necessary framework for cell attachment, proliferation, and differentiation, while also facilitating tissue organisation and the exchange of nutrients and waste products [16-19]. To further guide tissue regeneration, biological cues such as growth

factors (including bone morphogenetic proteins (BMPs) [20, 21], fibroblast growth factors (FGFs) [22, 23], vascular endothelial growth factor (VEGF) [24-26], and transforming growth factors (TGF- β) [27-29] are incorporated to modulate cellular phenotype and promote tissue-specific differentiation [30]. These elements are combined to create engineered tissues *in vitro* or *in situ*, aiming for tissue-specific engineered grafts. To build on the foundation of these 3 elements, the biofabrication field has emerged as a cutting-edge branch within TE, aiming to enhance precision, scalability and mostly the structural complexity of engineered tissues [31]. These methods are crucial for overcoming the limitations of conventional scaffold fabrication techniques, enabling the generation of constructs that closely mimic the intricate hierarchical structures of native musculoskeletal tissues [32, 33]. Traditional biofabrication strategies involved seeding cells within biomaterials or a 3D scaffold and tuning the cell phenotype with growth factor stimuli [34]. However, the use of supporting hydrogels or scaffolds can limit the ability of cells to interact and remodel the tissue. Therefore, scaffold-free approaches have emerged as a branch of developmental engineering in which key processes during embryo development are being recapitulated *in vitro* to generate more biomimetic constructs. Microtissues, organoids and cellular aggregates represent a promising scaffold-free approach. These aggregates act as biological building blocks that can fuse and reorganise into more complex, functional and multilayered grafts that closely resemble the native tissues. [33, 35]. Because microtissues can be fabricated in a high-throughput manner and are compatible with both bioassembly strategies and bioprinting, they represent a promising route to engineer highly biomimetic musculoskeletal tissues [36]. Within the broader domain of biofabrication, bioprinting has emerged as a particularly powerful and versatile technology [37]. By coupling the high-throughput generation of microtissues with bioprinting, it becomes possible to fabricate complex, heterogeneous structures with high reproducibility and spatial fidelity in a scaffold-free format, more closely mimicking native tissue architecture and function.

1.2 3D bioprinting for musculoskeletal tissue engineering

3D bioprinting offers significant advantages for musculoskeletal TE, where the intricate organisation of multiple cell types, ECM components, and mechanical gradients is critical for biomimicry and functional regeneration. 3D bioprinting is arguably the most

transformative biofabrication method, allowing for layer-by-layer deposition of cells and biomaterials (bioinks) to create complex, pre-designed 3D structures with high spatial control [38]. Extrusion-based bioprinting is the most common method, which involves extruding continuous filaments of bioink through a nozzle. It is suitable for printing high-viscosity bioinks and high cell densities, making it adaptable for various musculoskeletal tissues like bone [39] and cartilage [40], where cell-laden hydrogels are combined with mechanically robust materials. Such reinforcing structures can potentially be engineered using alternative additive manufacturing approaches, such as fused deposition modelling (FDM) and melt electro writing (MEW). Both these strategies enable the fabrication of porous scaffolds that can recapitulate the arrangements of collagen of the native tissue, such as cartilage [41, 42] and meniscus [43-45]. Light-based bioprinting (i.e. Digital Light Processing (DLP), stereolithography, volumetric printing) has been used to spatially pattern a cell-laden photocurable bioink/photo resins to spatially pattern cells in 3D constructs, offering very high resolution but limiting the size of the construct due to the limitation of the penetration of light into a deeper construct [46-53]. Musculoskeletal tissues—including bone, cartilage, tendon, ligament, and skeletal muscle—exhibit hierarchical architectures with region-specific variations in ECM composition, cellular phenotype, and mechanical strength [54]. Traditional scaffold fabrication methods (e.g., electrospinning, freeze-drying, or moulding) lack the spatial control necessary to reproduce these gradients [55]. As bioprinting enables the precise spatial deposition of multiple bioinks, cell types and bioactive molecules, it allows researchers to design constructs that replicate native tissue interfaces, such as the osteochondral unit or the myotendinous junction. By tuning the printing parameters, it is possible to control filament alignment, porosity and microarchitecture, thereby modulating mechanical anisotropy to match native tissue behaviour [56]. This feature is particularly relevant for AC, meniscus, ligaments, and muscles, where fibre alignment and load transfer govern function [57-60]. Moreover, bioprinting is compatible with patient-specific modelling, integrating imaging data (CT, MRI) to fabricate grafts tailored to individual anatomical geometries [61, 62]. It also facilitates the co-delivery of biochemical and biophysical cues, such as gradients of growth factors (e.g., TGF- β , BMPs, VEGF) [63] or controlled mechanical environments, to direct region-specific differentiation of stem or progenitor cells. In recent years, μ T-based and modular bioprinting approaches have further advanced the field by using preformed

cellular aggregates or spheroids that self-assemble and produce their own ECM, enhancing maturation and structural integrity [33, 36, 49, 64-66]. Together, these capabilities position bioprinting as a uniquely versatile strategy for engineering musculoskeletal tissues that require both biological fidelity and mechanical performance.

1.3 Objective of the thesis

There is a growing need for developmentally inspired TE strategies that can fabricate grafts capable of replicating the complexity of native musculoskeletal tissues. Current approaches often fall short, producing constructs that lack the hierarchical organisation, anisotropy, and functional integration required for successful regeneration. As discussed earlier, the use of cellular aggregates or μ Ts as building blocks presents a promising alternative for addressing this challenge. However, even when μ Ts successfully fuse, the resulting constructs typically fail to recapitulate the intricate architecture of native tissues.

The overall objective of this thesis is to develop a new 4D bioprinting platform capable of directing μ T fusion, differentiation and (re)modelling to engineer highly anisotropic, spatially organised musculoskeletal grafts. To achieve this goal, the thesis is structured around the following specific aims:

Aim 1: To direct the fusion of μ Ts at different maturation states and densities to engineer scaled-up grafts: The field of developmental engineering (DE) seeks to design new methods to fabricate *in vitro* grafts that can effectively regenerate anisotropic soft tissues such as cartilage and meniscus. μ Ts are promising building blocks for this purpose, but controlling their fusion and remodelling to generate scaled-up, structurally complex grafts remains a major challenge. In Chapter III, a two-fold strategy is proposed to address these limitations. First, the influence of μ T maturation state and density on their controlled fusion was investigated. Secondly, their ability to form large-scale grafts of clinically relevant sizes was investigated.

Aim 2: To 3D bioprint μ Ts in geometrically defined structures, to guide their self-organisation, resulting in the development of tissues with user-defined architectures. Despite the growing use of μ Ts as building blocks for complex tissue constructs, current

biofabrication strategies often lack control over their spatial arrangement and organisation within three-dimensional architectures. This limitation prevents the precise guidance of μ T fusion and remodelling, resulting in constructs that fail to reproduce the structural anisotropy and functional complexity of native tissues.

To overcome these challenges, Chapter IV focused on employing 3D bioprinting to spatially organise μ Ts into geometrically defined architectures, thereby directing their self-organisation into user-defined tissue structures. The development of this platform required careful consideration of the cell types used, their compatibility with bioinks and support materials, and the critical steps involved in μ T printing—from extrusion through a nozzle, to post-printing fusion, maturation, and phenotype maintenance. To address these aspects systematically, chapter IV consolidated the technical and biological principles underpinning effective μ T bioprinting. It identified the parameters that most critically influence printing fidelity, μ T integrity, and post-printing behaviour, translating them into practical design criteria for the development of reliable bioprinting workflows. By combining insights from literature with data generated in this work, a structured approach is established for selecting materials, tuning printing conditions, and evaluating construct performance in terms of fusion dynamics, differentiation potential, and phenotype stability. This integrated framework is intended to advance μ T bioprinting from an empirical practice toward a standardised and reproducible methodology, thereby supporting its broader use in regenerative medicine.

Aim 3: To develop a 4D bioprinting platform to spatial pattern μ Ts and enable the engineering of anisotropic musculoskeletal tissues: 3D bioprinting alone, traditional support baths, or standard 3D cell cultures strategies are often insufficient to reproduce the dynamic environment in which biological tissues develop. In native development, developing tissues form alongside others, which generate mechanical forces that influence morphogenesis. To emulate this, Chapter V developed a 4D bioprinting platform where MSC-derived μ Ts were printed within temporally adapting support baths. These baths, with different physical properties (mechanical and rheological properties), exerted compressive forces around the printed filaments over time in culture, creating a different microenvironment dependent on their respective physical properties. Such mechanical cues modulated the phenotype of MSC-derived μ Ts, supporting the formation of diverse

musculoskeletal tissues. This approach integrates the advantages of using μ Ts as building blocks, with additive manufacturing (AM) and clinically relevant cell sources (MSCs), thus establishing a translationally relevant foundation for future preclinical applications.

Aim 4: Development of a more biodegradable support bath to engineer a more clinically relevant, implantable and mechanically competent meniscal graft. Bioprinted tissues are rarely translated to preclinical studies, particularly in large defect models, because they often lack sufficient mechanical strength and structural anisotropy. These shortcomings are typically linked to the use of soft hydrogels as printing materials, limited ECM deposition and the poor organisation of collagen fibres within the constructs. Chapter VI focused on refining the previously developed support bath composition to improve both the structural and mechanical properties of the printed tissues. The goal was to fabricate anisotropic constructs with collagen fibres preferentially aligned along the long axis of the design, thereby enhancing the tensile properties of an engineered meniscus-like tissue in this direction. A critical aspect of this optimisation was achieving an appropriate balance between material degradation and tissue maturation. The support bath must not only sustain the printed construct during fabrication, but also gradually degrade to allow cellular deposition of new matrix and structural remodelling through inter-filament fusion. The influence of the temporal degradation of the bath on chondrogenic differentiation, structural organisation, phenotype maintenance, and biomechanical performance of the engineered tissues was investigated. This refined approach aims to generate bioprinted constructs with mechanical integrity and collagen organisation closer to those of native meniscal tissue, making them more suitable for preclinical implantation and functional testing.

Aim 5: Bioprinting regionally defined grafts through spatial growth factor patterning for meniscus tissue engineering. Despite major progress in meniscus TE, reproducing the native tissue's complex regional composition and organisation remains a significant challenge. This limitation stems partly from the use of a single cell type distributed homogeneously throughout constructs, which fails to mimic the zonal heterogeneity of the native meniscus. Building upon the previous aims, the final objective of this thesis is to combine spatial growth factor patterning with the established 4D bioprinting platform to engineer

regionally defined grafts. In chapter VII, MSC-derived μ Ts were exposed to either connective tissue growth factor (CTGF) or TGF- β 3, representing cues for the outer and inner meniscal regions, respectively. Using Laponite as a delivery vehicle for controlled and localised growth factor release, the aim was to induce region-specific differentiation within the same printed construct. This strategy enabled the creation of hybrid fibrocartilaginous tissues that resemble the native meniscus in both structure and function.

By integrating spatial control over biochemical signals with the anisotropic architecture provided by μ T-based 4D bioprinting, this thesis sought to produce musculoskeletal tissue grafts that mimic key aspects of the structural, compositional, and functional heterogeneity of the native tissue. Through the combined use of μ Ts, temporally adapting support baths, and spatial growth factor patterning, it provided a foundation for translationally relevant grafts suitable for future preclinical and clinical applications.

Chapter II. Literature Review

2.1 Introduction

Musculoskeletal TE aims to restore the structure and function of tissues compromised by disease (e.g., OA) or injury. Within the knee, multiple tissues coexist yet perform distinct roles integral to their intrinsic structure–function relationships. AC is a specialised connective tissue lining the ends of long bones; its depth-dependent composition and hierarchical collagen architecture confer load-bearing capacity and directional mechanical behaviour [67]. The menisci are wedge-shaped fibrocartilaginous structures that stabilise the joint and distribute axial loads, protecting the underlying cartilage [68, 69]. Knee ligaments are dense fibrous tissues that constrain translation and rotation, providing joint stability, while tendons connect muscle to bone and transmit force to produce movement [70]. The coordinated function of these tissues enables locomotion and athletic performance. The impairment of any single component can lead to pain, dysfunction, and progression toward OA. Preserving tissue integrity across a patient's lifespan is therefore essential.

Large or chronic injuries frequently exceed the limited regenerative capacity of musculoskeletal tissues, and current clinical interventions often fall short of restoring native architecture and function [11, 71]. Strategies such as autologous chondrocyte implantation (ACI) for cartilage [72] and meniscus repair, mesenchymal stromal/stem cell–based therapies for meniscus repair [73], and platelet-rich plasma injections for tendon pathology [74] can offer symptomatic relief or partial repair, but outcomes remain variable and depend on defect size, chronicity, and patient factors such as age and lifestyle [13, 75]. This therapeutic gap underscores the need for advanced regenerative approaches.

TE has pursued two complementary paradigms: top-down and bottom-up strategies. Top-down approaches combine defined cell populations with biochemical cues, instructive scaffolds, and mechanical stimulation to guide regeneration. In contrast, bottom-up strategies assemble complex tissues from modular building blocks, such as μ Ts or cell sheets, to better control structural features of engineered grafts [15, 76, 77]. μ Ts

preconditioned toward specific phenotypes have shown promise as biological building blocks for cartilage and meniscus repair, osteochondral regeneration, and large bone defects both *in vitro* and *in vivo* [41, 42, 64, 78-80]. However, these strategies fail to reproduce the complexity of the native tissue, and their limitations are the absence of control over the native tissues 'architecture for a more biomimetic graft. Here, bioprinting extends these concepts by enabling spatially controlled assembly of cells and/or μ Ts to recreate the architectural complexity of musculoskeletal tissues, including the arcade-like collagen organisation of AC, the circumferential and radial bundles of the meniscus, and the highly aligned fibre architecture of ligaments and tendons. By integrating μ T-based, bottom-up assembly with bioprinting and careful user-defined bioprinting design, it is possible to programme anisotropy, zonal composition, and hierarchy into engineered constructs. Such advances could accelerate the translation of modular, biomimetic grafts for preclinical evaluation and ultimately address the pressing clinical need for durable and successful knee joint repair.

2.1.1 The importance of matrix structure and composition for musculoskeletal tissues

The successful regeneration of musculoskeletal tissues necessitates the development of engineered grafts that faithfully replicate the heterogeneous and anisotropic structure and mechanics of the native tissue. Musculoskeletal tissues exhibit complex hierarchical structures, with varying cell types, ECM compositions, and mechanical properties [81]. This importance is especially pronounced in musculoskeletal tissues like bone, AC, meniscus, ligament and tendons, which acquire intricate architectures tailored to their specific biomechanical roles during skeletal development [11, 82]. The occurrence of pathologies associated with tissue damage highlights the need for TE approaches aimed at guiding the effective regeneration of these tissues.

The application of tissue-engineered constructs is considered one of the fundamental approaches for repairing damaged or lost tissue, and in some cases, even certain organs [82]. Despite continuous efforts, traditional approaches in the field of musculoskeletal TE have struggled to fully replicate the structure, composition, and functional properties of

desired tissues. Specifically, achieving *in vitro* engineered AC and meniscus with a native-like organisation has proven to be challenging [87, 88]. These tissues are predominantly composed of ECM, a highly organised network of structural proteins and bioactive molecules that dictate both the mechanical performance and the biological function of the tissue [83, 84]. The composition and spatial arrangement of ECM components vary markedly across tissue types and anatomical locations, reflecting their distinct physiological roles [85].

From an anatomical perspective: articular cartilage lines the surfaces of synovial joints, the meniscus cushions and stabilises the knee, tendons connect muscle to bone, and ligaments connect bone to bone. Despite their differences in form and function, they all rely heavily on the organisation and composition of the ECM to maintain tissue integrity and function under mechanical stress [86]. Collagen, particularly type I and type II, is the primary structural protein across these tissues and is critical in resisting tensile and shear forces [85]. Its alignment and hierarchical organisation are essential for biomechanical competence. As illustrated in Figure 2.1, imaging techniques at multiple scales provide insight into how collagen architecture underpins tissue function. Polarised light microscopy (PLM) and Scanning Electron Microscopy (SEM) reveal the depth-dependent zonal organisation of collagen in AC (Fig. 2.1 A), where fibers are aligned parallel to the surface in the superficial zone to resist shear, perpendicular in the deep zone to anchor the tissue and support compressive loads [87, 88]. In the meniscus (Fig. 2.1 B), PLM shows circumferential fibre alignment, while Transmission Electron Microscopy (TEM) highlights tightly packed collagen fibrils: together these features enable efficient load distribution and resistance to hoop stresses [87, 88]. Similarly, SEM of ligaments (Fig. 2.1 C) reveals highly aligned collagen bundles, which provide the tensile strength required to stabilise joints under uniaxial loading [93]. These multiscale structural features directly dictate the biomechanical behaviour of each tissue.

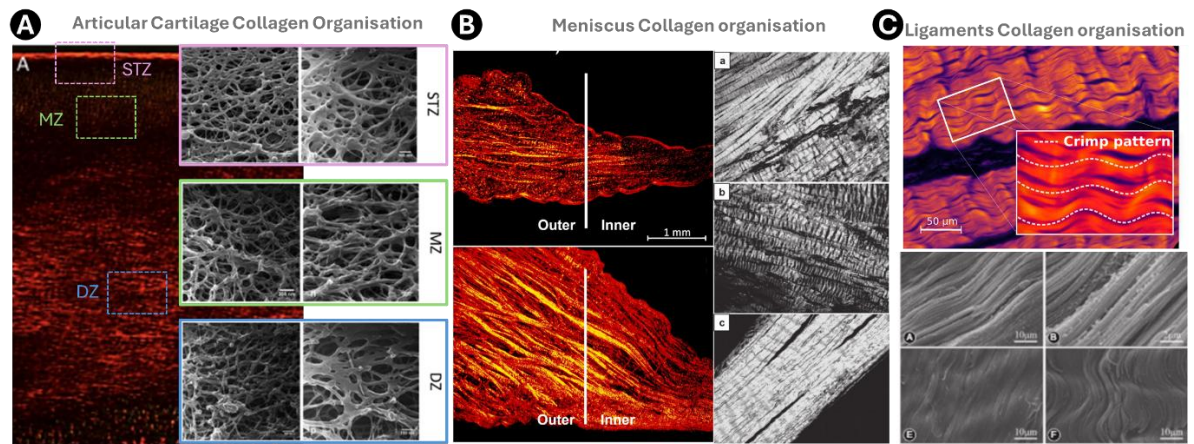


Figure 2.1. Specific collagen organisation in different musculoskeletal tissues. (A) Polarised light microscopy (PLM) images of articular cartilage stained with picosirius red, showing the superficial (STZ, pink), middle (MZ, green) and deep (DZ, blue) zones, each accompanied by corresponding helium ion microscopy images. (B) PLM image of collagen fibres in native meniscal tissue with corresponding higher-magnification transmission electron microscopy (TEM) images of collagen fibres. (C) PLM image of the anterior cruciate ligament (ACL) with corresponding scanning electron microscopy (SEM) images showing collagen bundles. The image was adapted from [89-93].

Any disruption in collagen architecture (i.e. type II collagen for AC)—whether through injury, degeneration, or poor-quality repair—can compromise mechanical function and predispose the joint to OA [94]. In addition to collagen, the ECM contains a complex mixture of proteoglycans and sulphated glycosaminoglycans (sGAGs) that provide compressive resistance and contribute to hydration and nutrient transport [11, 85, 87]. The type and concentration of these components vary by tissue: cartilage is rich in aggrecan, and GAGs such as chondroitin sulphate and keratan sulphate [95, 96]. In contrast, tendons and ligaments contain lower levels of proteoglycans and mostly rely on tightly aligned collagen bundles for tensile strength [97]. The balance and spatial distribution of these components are finely tuned to meet the functional demands of each tissue type. Regenerative TE strategies should also deliberately engage mechano-transduction pathways in a supportive manner. Biophysical features of the ECM, including stiffness, surface topography and the spatial organisation of the microenvironment, modulate cell behaviour [98-102]. Consistently, mechanical stimulation upregulates cartilage-specific mRNA expression of proteins such as collagen type II (COL2), aggrecan (ACAN), Sry-box transcription factor 9 (Sox9) and Cartilage oligomeric matrix protein (COMP) [103-106]. For

example, mechanically loading chondrocytes- or MSCs-laden hydrogels increases mRNA expression of cartilage-related genes (COL2, ACAN) [105, 107]. Conversely, mechanical unloading and cellular detensioning in tendons rapidly reduce alpha smooth muscle actin (ACTA2) and YAP/TAZ expression and increase catabolic enzyme production, leading to deterioration of tissue mechanical properties [108]. A deep understanding of how ECM components contribute to tissue function is essential for the design of regenerative therapies that can replicate the native biomechanics and durability of these complex load-bearing tissues.

2.1.2 The structure and function of Articular Cartilage

AC is a thin layer of connective tissue located above the subchondral bone with unique mechanical and physiochemical properties. The function of this tissue is directly related to its biochemical composition and to its intrinsic architecture [109]. In addition, these properties allow the tissue to (1) evenly distribute high loads; (2) act as a shock absorber; (3) act as a low-friction articular surface to facilitate the movement of the joint [110]. By looking at the tissue macroscopically, adult cartilage appears to be smooth on the surface [109]. The tissue consists mostly of water, ECM components and ~2% of chondrocytes [111]. Chondrocytes are the main cells populating the cartilage, which maintain the chondral physiological structure by secreting an ECM rich in type II collagen, proteoglycans, mainly sGAGs [111] whose function is to retain water in the tissue. Water serves as the most abundant component of AC, accounting for up to 80% of its wet weight [112]. A correct functioning of cartilage is facilitated by the flow of water through its structure and across the articular surface. This flow of water serves multiple purposes, including the transportation and distribution of nutrients to chondrocytes and the provision of lubrication within the joint [110]. In addition, the low cell density (compared to other tissues, such as bone) impairs the self-renewal of this tissue. Therefore, the regeneration of this highly specialised tissue still needs highly advanced strategies to overcome such problems, especially in TE approaches [113].

AC is composed of several different layers, in which chondrocytes have different morphologies related to the collagen fibres and their specialised function within the tissue [114]. Type II collagen is the main ECM component, along with other collagen types (Type VI collagen) and non-collagenous proteins (lubricin, decorin, etc.) [115]. The presence of

mainly type II collagen in the ECM allows the cartilage to adapt and tolerate a wide range of compressive and tensile forces to withstand heavy load, and protect the subchondral bone from any mechanical injuries [116]. AC is divided into four different regions; each region has a different composition (mostly in the collagen-type content), collagen fibrils orientation, cell density and morphology: (1) the superficial zone in contact with the synovial fluid and that contains chondrocytes and articular cartilage progenitor cells (ACPs) [117-119], (2) the middle zone with the presence of more spherical chondrocytes, (3) the deep zone and (4) the calcified zone, where the chondrocytes are undergoing hypertrophy [120]. Along these regions, the collagen fibre orientation changes from the superficial to the deep zone, creating an arcade-like structure, called the arcade of Benninghoff [121]. This complicated morphology and structure confer to the cartilage unique mechanical properties, allowing the knee to withstand the body weight and transmit load across the whole joint surface.

2.1.3 Tissue Engineering Strategies for Articular Cartilage Regeneration

Articular cartilage defects are still a challenge to regenerate. Injury or damage can lead to pain and joint dysfunction and ultimately to the development of OA. OA is a chronic and progressive disease that can significantly impact the functionality of one or more joints in the body. It presents with various clinical symptoms, such as pain, swelling, joint enlargement, and a reduced range of motion [109]. Currently, the gold standard for the treatment of severe OA is surgical options like total joint arthroplasty (TJA) [122]. None of the current surgical and emerging approaches leads to a total regeneration of the tissue. The microfracture method consists of removing damaged cartilage and inducing microdamage. This process aims to stimulate the MSCs from the bone marrow to migrate, proliferate and differentiate into chondrocytes and fill the defect region with new tissue [123]. This method can improve function and increase joint space in patients with full-thickness chondral defects [124]. Autologous chondrocyte implantation (ACI) consists of isolating chondrocytes from a non-damaged region of the AC on the knee joint. The chondrocytes are expanded *in vitro* and re-implanted in the lesion site after 10-20 days of *in vitro* expansion [125]. Peterson *et al.* (2010) report a long-term follow-up study where patients underwent ACI that showed good outcomes in most patients. After 10 to 20 years,

the ACI procedure seemed to be a long-term solution for patients with full-thickness lesions of the knee joint. Despite a few successes, the cartilage formed after ACI or microfracture is closer to a fibrocartilaginous type, which limits the clinical outcomes [123, 125]. These techniques often require the mechanical formation of other defect sites in the knee and a part of the donor's tissue. Therefore, there is still an urgent need of novel treatments for AC regeneration.

MSC-based TE could offer a more rational route by combining living cells with biomaterials, spatially controlled cues, and mechanobiology to recapitulate the structure and function of hyaline cartilage while addressing the inflammatory and mechanical environment of the joint. MSCs are attractive because they can undergo chondrogenesis, secrete trophic factors that modulate inflammation, and are amenable to scalable manufacturing [126, 127]. Unlike terminally differentiated chondrocytes, MSCs can be expanded more readily, and their secretome can protect the ECM from catabolic cytokines common in injured or osteoarthritic joints [128, 129]. Collectively, MSC-based approaches outperform chondrocyte-only implants, which are prone to dedifferentiation after monolayer expansion, and support a pro-regenerative environment [130]. MSCs can also be assembled into cellular aggregates, spheroids or μ Ts and used as modular building blocks for TE. Cellular aggregates are just condensed stem or progenitor cells [131]; μ Ts are condensed cells that have been differentiated specifically towards a phenotype through exposure of specific growth factors[36], while organoids are typically produced using embryonic stem cells (ECs), neonatal stem cells, or iPSCs and are capable of forming more complex tissues with different layers mimicking the native organs (i.e. lung organoids, intestinal organoids) [132]. Specifically, cartilage μ Ts self-produce collagen and glycosaminoglycans, fuse rapidly, and preserve a pro-chondrogenic phenotype that is often lost during extensive 2D expansion [42, 64, 66, 78, 133-135]. Using μ Ts as modular units also minimises the amount of foreign material, improving cell density and cohesiveness of the construct [33]. Scaffold-free or hybrid approaches that combine μ T fusion with a supportive, degradable framework have shown faster maturation and better collagen alignment than cell-in-gel strategies [42, 66]. 3D bioprinting could advance the field of TE and cartilage repair on two fronts: geometry fidelity and mechanical/biochemical gradients. First, bioprinting enables patient-specific grafts that match complex defect contours and thickness profiles, improving

construct stability post-implantation [88]. Second, bioprinting allows spatial patterning of mechanics and biochemical cues [136]. For instance, a softer superficial zone (AC) and a stiffer deep zone (bone) can be laid down in a single construct using hydrogels with different stiffness [137], while localised delivery of tissue-specific growth factors (such as TGF- β 1 or - β 3, BMP-2) can be embedded where needed [138-140]. Gene-activated 3D printed scaffolds can be loaded with short interference RNAs (siRNA) or micro RNAs (miRNA) encoding chondrogenic or anti-catabolic factors that transfect MSCs *in situ*. The result is sustained local production of cues like SOX9 or TGF- β , which boosts COL2/ACAN while limiting MMP13 and ADAMTS activity [141, 142]. Gene editing is also being explored to blunt hypertrophy (for example, manipulating RUNX2 or COL10A1 pathways) and to enhance resistance to inflammatory signalling. While these approaches face regulatory scrutiny, they directly address two persistent failure modes: loss of chondrogenic phenotype and matrix degradation under inflammatory load [143-146]. Therefore, coupling any of these strategies with a clinically relevant source could really advance the field of TE in the coming years, paving the way for new treatments for OA.

2.1.4 Meniscus

The menisci are a C-shaped tissue found between the femur and the tibia and are divided into medial and lateral menisci. Their location and function are essential for increasing the contact surface between the tibia and the femoral condyles to ensure a correct load distribution across the knee joint [68]. The meniscus tissue is divided into 2 main regions: 1) the outer zone and 2) the inner zone. The outer zone is 75-80 % of the meniscus tissue and is mostly composed of type I collagen with collagen fibres oriented circumferentially along the meniscus tissue [87]. This zone is partly vascularized. The inner zone is avascular, mostly composed of collagen type II (60%) and collagen type I (40%) and a moderate amount of sGAGs. The outer zone is mostly composed of fibroblast-like cells, while the inner zone has chondrocyte-like cells (fibrochondrocytes) that secrete ECM rich in GAGs [147, 148]. This complex structure and composition confer on the meniscus tissue its ability to ensure load distribution across the joint. In fact, the organisation of the collagen fibrils confers to the meniscal tissue a unique collagen microarchitecture where the presence of circumferentially aligned collagen fibrils and radially aligned, defines the meniscus tissue as

an anisotropic tissue, with a higher tensile modulus in the circumferential direction (100-200 MPa), than in the radial direction (50-60 MPa) [59, 60, 87, 147, 148].

2.1.5 TE strategies for Meniscus

The meniscus is frequently injured, and, because of its limited intrinsic healing capacity, tears often require medical or surgical intervention. One of the most common treatments is meniscectomy for degenerative tears in the avascular inner zone, involving partial resection or removal of the inner meniscus. This procedure can alter joint biomechanics and load-bearing, accelerating inflammation and the onset of OA [149]. Emerging alternatives include implants fabricated from natural or synthetic polymers (for example, polyurethane scaffolds such as Actifit®) and cell-based therapies delivering reparative populations, particularly MSCs [72, 80, 125, 150-152]. MSCs have been administered via direct injection and in combination with biomaterial scaffolds, including hyaluronic acid [153] or collagen-based scaffolds [73]. Across preclinical and early clinical studies, MSC-based interventions appear safe and can improve repair tissue quality compared with conventional management. Nevertheless, these strategies may still fall short of engineering a mechanically competent meniscal graft because they do not reproduce the native zonal organisation. 3D printing can address this need by enabling spatial control over cells and biochemical cues.

Because of the nature of the meniscus, composed of different regions, multimaterial, dual-cartridge printing could support region-specific differentiation of the engineered grafts tailored to the outer fibrocartilaginous and inner cartilaginous zones. For example, a scaffold could be functionalised with growth factors or with microspheres loaded with different growth factors in different zones to drive MSCs differentiation [154, 155]. Recently, a migratory cell population has been identified that mobilises from the meniscus to injured regions of the joint and contributes to tissue repopulation [156, 157]. These meniscus progenitor cells (MPCs) can be isolated using a fibronectin-adhesion assay; although a definitive immunophenotype has not yet been established, MPCs have already been employed in TE strategies to recapitulate the zonal organisation of engineered meniscal grafts [44, 78]. However, the yield of MPCs from meniscal tissue, which may also be inflamed after injury, is relatively low, so MSCs remain an attractive cell source for meniscal

repair. Combining MSCs, obtainable from bone marrow, adipose tissue, synovial fluid, and other tissues, with 3D bioprinting enables spatial control over cell placement, hydrogel stiffness gradients, and growth-factor patterning to create mechanically competent grafts that reproduce native structural features. Together, bioprinting integrated with *in vitro* recapitulation of embryonic morphogenesis offers a path to grafts that more faithfully reproduce the structure and biomechanics of native musculoskeletal tissues, including AC and the meniscus.

2.2 Modular Tissue Engineering: μ Ts as building blocks for musculoskeletal TE

Musculoskeletal tissues of the knee joint are often injured, which can lead to progressive cartilage loss and loss of tissue physiological function. As these tissues lack the inherent capacity to effectively heal injured lesions, achieving complete regeneration of such tissues remains a significant challenge [4]. TE strategies aim to fabricate constructs *in vitro* that resemble the morphology and biochemical properties of native tissue, based on the assumption that such impacts promote superior regeneration *in vivo*. Two primary approaches are employed in tissue regeneration: 1) top-down and 2) bottom-up approaches, with the latter also known as modular TE [35]. In the first approach, also called classical TE, cells are encapsulated in a hydrogel or seeded on the surface of a 3D scaffold, and these constructs are then stimulated to promote the deposition of tissue-specific ECM. This allows cells to proliferate and differentiate within the three-dimensional network created by the scaffold itself. On the other hand, the bottom-up or modular TE approach aims to engineer a modular unit on a microscale, which can be assembled to biofabricate a more complex tissue [35]. The objective of modular TE is to mimic the structural and functional units found in nature, such as the osteon in bone, the nephron in the kidney, or the lobule in the liver [158-160]. In TE, these modular units (μ Ts, organoids, aggregates, cell sheets) can be assembled to control the geometry and composition of the final engineered construct compared to top-down approaches. These modular units can be brought together and assembled in a more direct way (confinement, surface energy) or in a controlled manner through AM techniques such as bioprinting. μ Ts have recently

developed a lot of interest among researchers because they emulate mesenchymal condensation, preserve cell phenotype and accelerate ECM deposition compared to single cell strategies [36, 161]. Their mechanical integrity, as well as their modular nature, helps manage diffusion limits for scalable constructs. Therefore, μ T could be part of a new versatile high-throughput platform to build biomimetic and scaled-up tissue grafts. The hope is that bottom-up approaches in TE will allow the final engineered graft to exhibit a structure and composition that closely resemble the native tissue.

2.2.1 Microtissues for engineering articular cartilage

Modular TE for cartilage regeneration aims to mimic the cellular condensation processes happening *in vivo* without the use of a scaffold. During cartilage formation, the condensation is the first stage before the commitment of the MSCs to differentiate into prechondroblasts [82, 162]. TE strategies that resemble this stage are emerging as optimal platforms compared to traditional TE approaches. Strategies that support cellular aggregation and μ T formation have been shown to support a superior chondrogenic phenotype [163]. Pioneering work from Bhumiratana *et al.* (2014) demonstrated the ability to regenerate functional cartilage from human MSCs, with physiological composition and biomechanical properties. Such μ Ts were able to fuse and differentiate in a centimetre-sized piece of cartilage-like tissue [164].

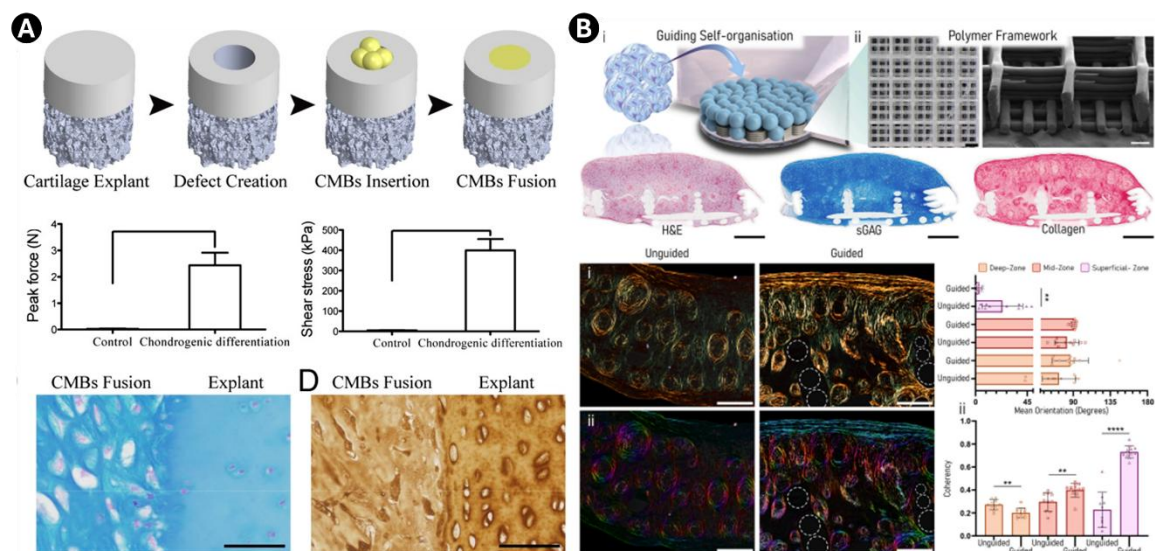


Figure 2.2 Microtissues are widely used as biological building blocks due to their capacity to fuse and self-organise. (A) Microtissues are introduced into a cartilage explant, where they fuse and develop into a biomimetic tissue that is well integrated with the native explant. (B) Microtissue self-organisation is guided by the design of polymeric microchambers, enabling the engineering of aligned cartilaginous microtissue-based constructs. Image was adapted from [64, 164].

Because of the poorer mechanical properties achieved so far, in this latest work from Burdis *et al.* (2022), μ Ts have been seeded into a PCL-based scaffold printed with FDM for joint resurfacing, where hypertrophic cartilage μ Ts pre-vascularised are used as bone precursor and localised just below cartilage μ Ts (on top). This strategy has been implanted into an osteochondral defect, and after 6 months *in vivo*, it supported the restoration of a more normal AC surface [42]. However, a limitation of this work is the fact that we still can't reach the same anisotropic structure of the native tissues, so new methods of directing the alignment of the collagen network in engineered constructs is important. One step has been done with enzymatic treatments, such as chondroitinase ABC (cABC) treatment for cartilage μ Ts to get better alignment of the collagen network during tissue maturation [165]. This, however, still doesn't fully recreate the organisation of the collagen network in AC and the manual deposition of the μ T inside these polymeric scaffolds also impairs and prevents the control on the precise and controlled deposition of the μ Ts. Therefore, new strategies to spatially pattern μ Ts in a controlled manner are needed for cartilage TE.

2.2.2 Microtissues for engineering meniscus

The meniscal tissue is formed from the mesenchymal condensation during the early stages of embryonic development, similar to cartilage and ligaments. Therefore, developmental engineering paradigms can be applied as well regarding meniscal regeneration. Recent studies have identified meniscus resident cell populations, analogous to articular cartilage resident cells, and demonstrated their use in tissue-engineering strategies to generate meniscal tissue *in vitro*, with preliminary *in vivo* investigations underway [118, 157]. Numerous studies have investigated meniscus-derived spheroids and μ Ts for novel TE strategies. Owing to their intrinsic capacity to fuse and function as modular building blocks, meniscal μ Ts represent a promising source for construct assembly, with resident progenitor cells offering a population that closely reflects native fibrochondrocytes [166].

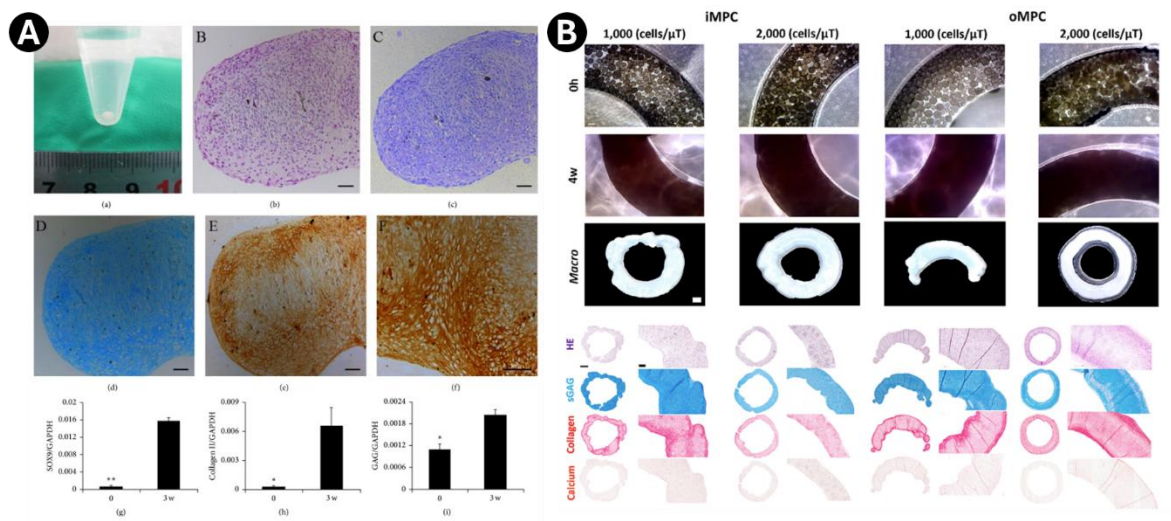


Figure 2.3. Meniscus-derived cells are isolated and used to generate fibrocartilaginous microtissues for meniscus tissue engineering. (A) Mesenchymal stromal cells (MSCs) obtained from meniscal tissue debris are isolated and self-assembled into pellets that undergo chondrogenic differentiation, as shown by sGAG staining and collagen type I staining. (B) Meniscus progenitor cells isolated from meniscal debris are used to recreate zonally organised meniscal tissues, exhibiting phenotypic differences between the outer and inner zones. Image was adapted from [78, 166].

Prior work has cultured meniscus-derived spheroids under varying oxygen tensions and shown that low oxygen ($\approx 5\%$) enhances redifferentiation and ECM deposition [167]. In addition, meniscus-derived spheroids have been embedded within hydrogels to examine cell migration in continuous networks, informing TE strategies for meniscal repair [168]. Recent work has identified meniscal progenitor populations and leveraged them in TE

strategies to bio assemble region-specific constructs. The approach isolates progenitor cells by physically dissecting the outer and inner meniscal zones and harvesting each population separately. When assembled, these zone-matched progenitors effectively recapitulate the distinct extracellular matrix profiles of the two regions, with clear differences in collagen-specific deposition [78].

Despite this promise, several practical barriers limit their near-term clinical utility: autologous donor tissue is scarce, cell yields are low, and extensive *ex vivo* expansion diminishes progenitor potency. In contrast, MSCs are comparatively abundant, can be sourced with less morbidity, and are supported by a broad literature showing efficacy in meniscal tissue engineering across biomaterial and bioprinting platforms. Taken together, these considerations position MSCs as a clinically relevant and scalable cell source for meniscal repair, contingent on implementing instructive cues that direct stable, zone-appropriate fibrocartilaginous phenotypes and mitigate hypertrophy.

2.2.3 Directing the fusion of multiple microtissues: new strategies.

Engineered cartilaginous tissues have already been successfully formed by bringing together cellular aggregates or μ Ts, allowing them to self-assemble, fuse and reorganise into a more functional construct [164, 169]. While the fusion of μ Ts to create a scaled-up tissue is a valuable approach, it is important to acknowledge that the resulting architecture of the graft may not closely resemble the native tissue structure. This discrepancy between the engineered graft and the native tissue architecture is still a challenge. Achieving a fully native-like structure remains a complex goal that requires further research and advancements in the field. Challenges in directing the formation of a superior phenotype are associated with 1) the discovery of new biomaterials or 2) different bio-fabrication strategies that can support and direct the fusion and remodelling of the μ Ts to engineer anisotropic tissues. To achieve a cohesive tissue, a homogeneous and complete fusion of the individual μ Ts is required. In other words, the successful fusion of μ Ts involves the complete integration of the cells and the ECM components to create a more cohesive tissue structure [164]. This fusion process should eliminate any structural remnants from the original μ Ts, resulting in a unified tissue construct [170, 171]. Nilsson *et al.* (2021) explored

the tissue fusion of cartilaginous μ Ts in the context of endochondral bone formation. Different degrees of maturation of the single μ Ts before fusion were explored, showing that the fusion capacity of the μ Ts is superior when the μ Ts are less mature [172]. ECM production can vary when the μ Ts are brought together: the higher the amount of ECM produced prior to fusion, the more limited is the fusion between μ Ts. Bhumiratana *et al.* (2014) explored the concept of the time of fusion of the μ Ts and how it can influence the final cartilaginous phenotype. In their work, it has been shown that the μ Ts set tenascin boundaries after 7 days of growing independently before fusion. The presence of these boundaries might impede the homogeneous fusion of the tissue [164]. De Moor *et al.* (2020) showed the fusion of single μ Ts after 7 days in culture in chondrogenic media. Although the μ Ts have successfully fused, the resulting tissue does not appear completely compact after a three-week culture period, and the tissue is not suitable for implantation [173].

To the best of our knowledge, in the TE field, the functional fusion of μ Ts to engineer a human-sized cartilaginous graft has not been achieved yet. One of the reasons is that the mechanisms of the fusion of μ Ts are not fully understood yet. Several engineering techniques have been developed to induce μ T fusion. Some of them involve physical forces like the hanging droplet merging method. Here, each μ T is in a single droplet to allow the self-assembly, and afterwards, two droplets are merged to allow the fusion of two μ Ts [174]. Another method uses a quantitative high-throughput platform to semi-automatically select doublets and automatically monitor their fusion kinetics. The automated selection makes the fusion process easier and more precise when compared to a more manual technique [175]. Engineering a cartilaginous graft with μ Ts as building blocks is an inherently scalable process by using biofabrication platforms. In the “Kenzan method”, such μ Ts are skewered into needle grids and allowed to fuse in culture. Although the viability was high during the process, the cells are not able to produce an ECM rich in collagen II GAGs. The result was a tissue that, when implanted *in vivo*, does not show any improvement in the cartilage structure [176]. Despite this, the study raises some questions about the final collagen structure within the tissue. Without any instructions, the cells *in vitro* are not able to resemble the native-like architecture. Daly *et al.* (2019) showed that cellular organisation can be directed within a 3D-printed polymeric framework. In their study, the framework acted as a physical boundary that guided functional maturation, yielding cartilage tissue

with zonal collagen architecture resembling native articular cartilage [177]. Similarly, Burdis *et al.* (2022) achieved aligned collagen by using μ Ts rather than single cells; the polymeric framework oriented collagen fibres along the PCL struts [165]. Because manual seeding limits the precise placement of μ Ts, 3D bioprinting provides a practical solution by enabling spatially defined deposition and control over their arrangement. By directing μ T placement and orientation, 3D printing can promote the development of targeted collagen architectures with improved structural organisation and closer resemblance to native tissues [43-45].

2.3 Developmental tissue engineering: recapitulating tissue development and formation *in vitro* to generate native-like tissue grafts

Developmental TE reframes tissue engineering in a non-conventional manner from constructing tissues to instructing them: it leverages cells' intrinsic capacity for self-assembly and self-organisation, while imposing boundary conditions that emulate the biomechanical processes of tissue development. Here, self-assembly denotes spontaneous cellular organisation without external forcing, and self-organisation describes the emergence of ordered structure through coupled cell–cell and cell–ECM interactions [178, 179]. Recreating these processes *in vitro*, particularly mesenchymal condensation and subsequent chondrogenesis, remains a central objective [180], motivating widespread use of μ Ts as modular building blocks across applications.

Biofabricated constructs can be theoretically steered toward native-like architecture when the size, shape, and geometry of the graft are deliberately constrained and when oxygen and nutrient diffusion gradients are allowed to develop in a controlled fashion, both of which strongly influence phenotype [181, 182]. In DE, multicellular self-organisation is coupled with guiding structures to apply spatially and temporally defined boundary conditions (for example, confinement, curvature, stiffness discontinuities, or prescribed load paths) to the forming tissue to direct tissue self-organisation (Fig. 2.1) [183].

These constraints mimic how, *in vivo*, neighbouring tissues shape a developing organ by exerting traction, compression, and shear rather than leaving it to form in isolation. The mechanical boundary constraints determine the directions of principal strain, where the contractile forces are used to model the tissue microarchitecture [184]. These contractile forces then drive morphogenesis from layer rearrangements during embryo development (gastrulation) to the development of musculoskeletal tissues. Here, collagen deposition aligns with the imposed constraints, progressively establishing the anisotropic architecture characteristic of mature musculoskeletal tissues [185-188]. When these mechanical forces are coupled with molecular cues (i.e. growth factors), a specific phenotype and tissue organisation and remodelling can be achieved. *In vitro*, several growth factors signalling pathways impact tissue development, such as TGF- β superfamily, BMPs and FGF. For example, TGF- β signalling secreted by muscles in response to muscle contraction is important to regulate tenocyte morphogenesis and ECM production [70]. Similarly, mechanical cues (stiffness, cell tensions) tune the mechanosensitive pathways of YAP/TAZ modulating BMP-2 expression [189, 190]. Consistent with this paradigm, self-assembly strategies have produced grafts with mechanical properties approaching those of native tissue [42, 164, 177, 191]. By integrating μ T-based assembly strategies- or modular TE- with engineered boundary conditions, it becomes possible to program, in space and time, the hierarchical structure of musculoskeletal tissues. Mimicking developmental rule sets through boundary-driven guidance, therefore, offers a credible route to engineered musculoskeletal tissues that more closely capture native form and function.

2.4 Physical and mechanical constraints as boundaries to engineer structurally organised tissues

Cellular self-organisation governs how cells arrange within polarised tissues during embryogenesis and continues to operate in adult tissue renewal. In development, tissues form alongside neighbouring structures that mechanically confine, compress or tension them, shaping morphogenesis [192]. Cells retain this sensitivity *in vitro*: when cultured within defined physical boundaries, they detect and respond to geometric and mechanical cues through integrin-mediated adhesions and cytoskeletal tension. These cues are

transduced into intracellular signals that regulate cytoskeletal organisation, cell polarity and ultimately alignment.

As a result, cells and μ Ts tend to align along the direction of principal stresses or within geometrical constraints that minimise internal tensions. For example, when confined within anisotropic geometries, cells elongate and organise their actin cytoskeleton along the scaffold-specific geometry, which, in turn, guides ECM deposition in the same orientation. This feedback between cell-generated forces and matrix remodelling enables local alignment to propagate across the tissue, generating an organised tissue [181-192].

Therefore, by imposing these cues *in vitro* in space and time, we can modulate cellular self-organisation to build constructs that recapitulate internal architecture, composition and function. The size, geometry and boundary conditions applied to a culture establish morphogen and force gradients that determine final tissue structure [181]. The self-organisation process that builds cellular aggregates requires only minimal external cues and growth factor stimulation. This process can already generate great levels of tissue organisation that cannot be matched by traditional scaffold TE [193]. In the case of more complex tissues, for example, colonies constrained on ECM micropatterns align with the boundary shape, and this local alignment can propagate over long distances to yield organised tissue-level patterns. Similarly, when intestinal stem cell-derived organoids or epithelial cells were seeded onto a microfabricated collagen scaffold, the imposed 3D topographies guided the spatial organisation by directing cell positioning [194]. In a related strategy, shaped epithelial monolayers with predefined crypt-like geometries were combined with controlled morphogen gradients across the tissue, reinforces spatial patterning and functional organisation, recapitulating key features of colon homeostasis [195].

In musculoskeletal systems, these principles are strongly linked to mechanobiological regulation. In cartilage, stiffness-gradient hydrogels provide a continuous mechanical that tunes how embedded chondrocytes or MSCs proliferate, adopt distinct morphologies, and deposit ECM, leading to a zonally organised tissue [196]. In osteochondral repair, zonally microstructured PCL scaffolds with distinct pore architectures in the cartilage and bone regions guide cell infiltration and ECM deposition so that cartilage-like and bone-like tissues self-organise within their respective compartments [197]. For skeletal muscle,

microgrooved hydrogel substrates constrain the directions available for cell spreading and fusion, driving myoblasts to align, fuse, and mature into parallel, contractile myotubes [198].

Together, these systems demonstrate that physical boundaries do not simply confine cells, but actively direct their behaviour by modulating force transmission, cytoskeletal organisation, and downstream signalling pathways. These principles make engineered physical cues integral to creating structurally complex tissues *in vitro*, including musculoskeletal tissues. By controlling these parameters, engineered systems can harness intrinsic self-organisation processes to generate structurally and functionally organised tissues [81, 181].

2.5 3D printing strategies to engineer anisotropic tissues

One of the major challenges in current TE strategies is that the new scaffold or engineered tissue is generally lacking the complex anisotropic features of native tissues. 3D printing can hold great promise in engineering biomimetic, highly organised musculoskeletal tissues. Engineering anisotropic soft tissues requires controlling the microstructure and inserting guiding structures during or after fabrication. One effective way is printing cells into a scaffold, where a mechanically robust polycaprolactone (PCL) lattice (often produced by MEW) sets fibre orientation and pore geometry and the piezoelectric inkjet head deposits MSCs into predefined pores to spatially pattern cells (Fig. 2.4 B). This strategy enables the generation of cellular gradients, for example, by varying printing parameters across different scaffold regions. In addition, the patterning of specific molecular cues, or the regional functionalisation of the scaffold with such cues, can enhance cell–scaffold interactions and guide ECM alignment for the engineering of anisotropic grafts [45, 199]. Nonetheless, these approaches are frequently limited by challenges associated with the long-term degradability of the scaffolds.

Among the various 3D bioprinting methods, extrusion-based bioprinting has emerged as one of the most versatile techniques due to (1) the wide range of compatible biomaterials, (2) relatively low cost, and (3) printing flexibility [200]. Bioprinting in air has been employed for patterning cells, biomaterials and/or soluble cues to fabricate organised tissues (Fig. 2.4

D). However, this technique often lacks a good resolution and only a narrow range of materials can be printed, such as bioinks with relatively low viscosity. When low viscosity inks are printed on a flat surface, they cannot retain their shape, often resulting in poor printing fidelity, leading to the collapse of the bioprinted construct [201]. This limitation can be addressed with new techniques to improve the shape fidelity and to create more complex structures, such as embedded 3D bioprinting. In the past years, this technique attracted the TE field, and more different embedded printing techniques have been developed. Here, the bioink is extruded within a secondary hydrogel- as a support bath- that serves as a temporary, reversible, biocompatible support [202]. The printing bath supports the bio ink during the printing process and prevents the final printed construct from collapsing on itself. Wu *et al.* (2011) first demonstrated this method by printing a sacrificial ink within a photocurable Pluronic F127 (diacrylate) support bath [203]. Afterwards, Hinton *et al.* (2015) developed the Freeform Reversible Embedding of Suspended Hydrogels (FRESH). The FRESH bath is made of gelatin microparticles that act as a Bingham plastic that maintains its viscosity at low shear stress and decreases it at higher shear stress. The FRESH platform allows the deposition of low viscosity hydrogels in a complex structure within a secondary supporting bath that helps maintain the final structure during printing, improving the print fidelity. Moreover, by being thermo-reversible, the bath melts at 37°C, allowing for washing out the final printed construct and making it suitable for cell culture [202]. The Suspended Layer Additive Manufacturing (SLAM) is another embedded 3D printing technique that aims to overcome some limitations of the FRESH 3D printing, such as the need for strict temperature control. Here, a fluid-gel matrix formed by a 0.5% (w/v) solution of agarose is used as a support matrix for low viscosity inks [204]. The SLAM offers a great set of advantages compared to FRESH: 1) temperature sensitivity, 2) bath transparency (user-friendly), 3) low cost, 4) reproducibility. Embedded bioprinting can also be framed as printing directly into a “living bath” of cells. In the Sacrificial Writing Into Functional Tissue (SWIFT) method, an acellular bioink is printed into a densely cellular matrix composed of Matrigel and collagen I mixed with spheroids. The living bath is first compacted by repeated centrifugation; the acellular ink is then deposited to create sacrificial paths that become perfusable channels within the matrix. This approach employs very high cell densities, and the resulting constructs sustain long-term perfusion, indicating functional tissue support [205]. The techniques described above,

along with related approaches, still face challenges in achieving robust architectural organisation in the final printed tissue. To address this, Liu and colleagues developed Filamented Light (FLight) biofabrication, which uses directed light to photopolymerize a photoactive resin into defined patterns (Fig. 2.4 C). This approach rapidly generates hydrogels composed of unidirectional microfilament networks with cell-scale diameters. Via optical modulation instability (OMI), an incident beam splits into FLight beams that trigger localised polymerisation and increase the local refractive index, creating self-focusing waveguides that extend polymerisation into long hydrogel microfilaments [206]. The FLight technique shows strong potential for producing grafts that provide guidance cues for *in situ* regeneration of aligned tissues, including nerves, muscle, cartilage, and tendon.

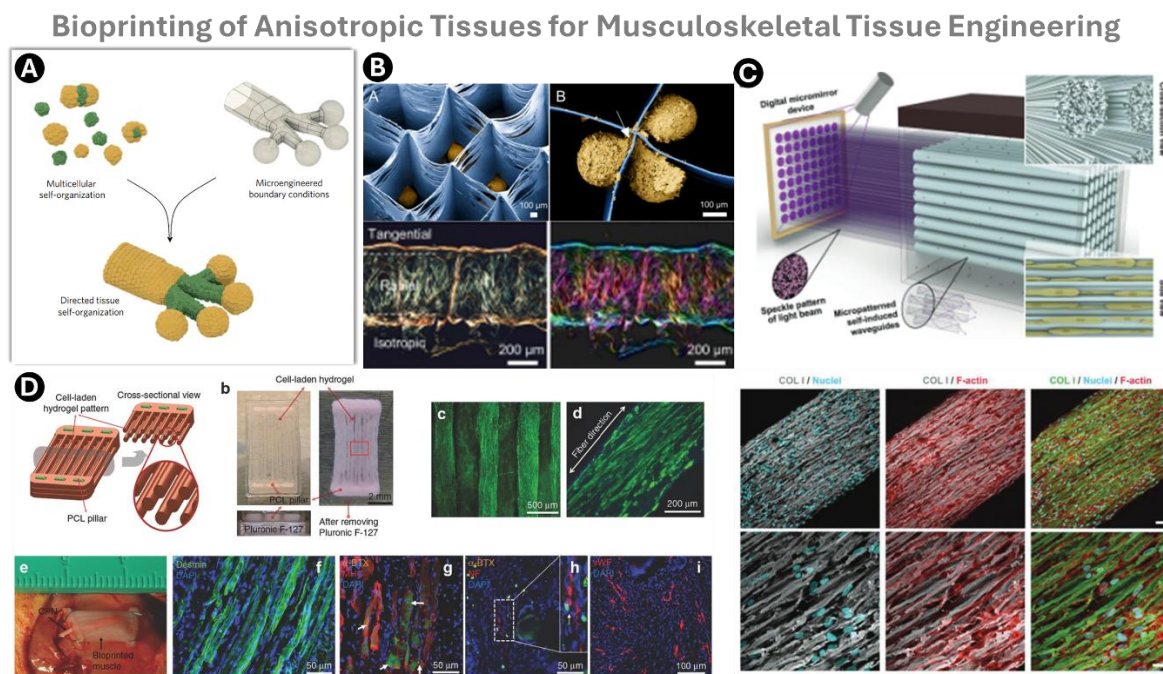


Figure 2.4. Bioprinting strategies for the engineering of anisotropic tissues via physical cue guidance. A) Schematic illustrating how imposed boundary conditions can be used to direct the self-organisation of cells or cell aggregates during tissue maturation. B) MSCs are deposited by inkjet bioprinting into polymeric microchambers that act as boundary conditions; the printed cells assemble into aggregates, adhere to the polymer surface and remodel the newly secreted ECM along these physical cues. C) In a FLight bioprinting approach, cells are encapsulated within a photoresin (i.e. GelMA) and, via OMI, boundary conditions are created at the microscale that guide cell alignment. D) Bioprinting of skeletal muscle constructs using physical constraints to generate functional grafts with enhanced anisotropy. Image was adapted from [45, 81, 206, 207].

Nevertheless, all these approaches present distinct limitations. In inkjet bioprinting that employs PCL scaffolds (Fig. 2.4B), the polymer's very slow degradation rate and its tendency to provoke a pronounced foreign-body reaction can favour robust immune activation and the formation of fibrotic capsules, driven by an exaggerated cellular and wound-healing response. In parallel, the SWIFT technique faces constraints in scalability: the very high tissue densities it achieves demand an extensive, perfusable vascular network to sustain oxygen and nutrient delivery and to avoid tissue necrosis, and the resulting constructs typically lack well-defined zonal organisation. With respect to embedded printing, although these platforms have enabled the printing of collagen-based bioinks, there are still no compelling demonstrations of highly aligned, viable, and truly scaled engineered grafts produced using these methods. These shortcomings need to be addressed, and one route is to integrate DE principles with modular TE strategies. Specifically, 3D printing μ Ts offers a means to preserve and mimic endogenous ECM production while coupling this with scalability and modularity; bioprinting μ T units should facilitate high-throughput fabrication of printed constructs that can be assembled with precise spatial patterning of these biological building blocks. Within this paradigm, the critical elements of DE would guide cells toward highly aligned organisation by combining mechanical constraints with carefully defined boundary conditions and temporally and spatially controlled molecular cues (for example, growth factors), thereby directing the fusion, maturation, and remodelling of μ Ts into scaled-up anisotropic tissues.

2.6 3D bioprinting strategies to spatially pattern μ Ts

A novel approach in TE for creating musculoskeletal tissue constructs involves a modular concept inspired by developmental biology. This concept recognises that more complex tissues are composed of repeating functional units or biological “building blocks” [35]. Through self-assembly and therefore fusion, these units are combined to create larger tissues and form a spatial arrangement of the cells mimicking the native architecture through cell-cell and cell-to-ECM interactions. To address cartilage defects, μ Ts can be injected instead of a single-cell suspension, as the latter often leads to inferior fibrous tissue due to dedifferentiation. μ Ts undergo maturation *in vitro*, creating a tissue-specific ECM due to their high cellularity and density. Notably, chondrospheres are an example of such

μ Ts used in clinical trials. These chondrospheres, ranging from 500 to 800 μ m in size, are generated from autologous chondrocytes [208]. Following injection, chondrospheres naturally adhere to the cartilage defect and subsequently assemble and randomly fuse [209, 210].

Instead of relying on μ T injection and non-directed fusion of μ Ts, an alternative approach involves utilising AM technologies to precisely assemble the cellular building blocks in a directed manner. This method allows for high spatial control, enabling the creation of complex and pre-designed configurations. By utilising 3D printing, tissue engineers can arrange the cells in space and create a desired structure with specific geometries and spatial organisation. This level of control enhances the reproducibility and customisation of tissue constructs. However, literature is scarce on the topic of 3D printing μ Ts. The process of 3D printing μ Ts remains challenging due to various factors, such as 1) the presence of high shear stresses during the printing process, (2) the viscosity of the ink used, (3) the requirement for appropriate supporting materials, and (4) the need to achieve a high cell density to allow the μ Ts to fuse. De Moor's work demonstrates the printing of chondrogenically primed μ Ts within a photo-crosslinkable hydrogel (gelMA). However, the resulting construct does not achieve a high enough μ T density necessary for the regeneration of mechanically functional tissue. The μ T density is insufficient to promote fusion between the μ Ts, resulting in a final construct that exhibits regions of high μ T density alongside regions lacking μ Ts altogether [173]. To address the issue of high shear stresses on cells when printing μ Ts at high density, Trikalitis *et al.* (2022) have introduced a new approach. Their concept involves printing μ Ts or cells in a liquid suspension at lower μ T densities. In this approach, the liquid suspension used for printing μ Ts, or cells, does not exhibit rheological behaviour typical of a bioink but behaves as a Newtonian fluid. Unlike bioinks that experience changes in shear stress and viscosity, the liquid suspension maintains a consistent viscosity and does not undergo significant alterations in response to shear stress. As a supporting material, a Xanthan gum-based bath is used as it absorbs the liquid component of the ink, resulting in the concentration of the bioink at the site where the cells are printed. By concentrating the bioink in the specific region where the cells are deposited, this technique ensures that a higher concentration of cells is present in the targeted region where printing takes place. As a result, optimal tissue fusion can occur within the final construct [211]. This innovative approach improves the chances of

successful fusion and integration of the printed μ Ts, addressing the challenges associated with high shear stresses and promoting the formation of a connected tissue structure. Scalzone *et al.* (2024) reported the bioprinting of dermal μ Ts into a Pluronic support bath. The printed constructs maintained high post-printing viability; however, the study did not demonstrate tissue anisotropy, a key requirement for engineering aligned tissues. Moreover, the use of a relatively large 18-gauge needle is suboptimal for achieving the micro- to nanoscale organisation needed for anisotropic tissues [212].

Despite meaningful progress, the available studies—including those discussed above—have not yet produced final printed tissues with the level of structural organisation required for robust tissue function. In most cases, the constructs show limited anisotropy and only partial μ T fusion, resulting in a discontinuous ECM with suboptimal fibre alignment. Fusion between μ Ts remains modest, likely reflecting limitations in precise patterning, suboptimal local mechanics, and insufficiently coordinated biochemical signalling during post-print maturation. There is, however, considerable room for improvement. A promising direction is to develop approaches that actively guide μ T fusion while exerting tighter spatial control over μ T arrangement within the printing bath. Strategies could include higher printing resolution, tuning of support-bath physical properties and post-print mechanical conditioning to promote tissue alignment. Advancing these elements should enable the construction of highly aligned, mechanically competent musculoskeletal tissues and, in the specific case of AC or meniscus, support regeneration of a zonally organised, functionally relevant graft. Therefore, each element critical to μ T bioprinting requires careful consideration, including bioink formulation, support-bath modification, the density of μ Ts within the ink, and post-print maturation. These factors are examined in the final section of this chapter and are addressed experimentally in this thesis.

2.6.1 Key components for embedded 3D bioprinting of μ Ts

- **Bioink**

Unlike traditional extrusion-based bioprinting, where immediate ink recovery post-printing is essential, embedded bioprinting leverages the rheological properties of the support bath to maintain the printed geometry, allowing for the use of softer, more cell-friendly bioinks

[213]. In this context, the shear-thinning properties of the bioink are highly important to ensure a rapid, smooth extrusion through the nozzle while minimising shear-induced cell damage. As the bath provides structural support, bioinks can be designed with higher water content and lower viscosity [214, 215]. This could provide advantages in the period post-printing (*in vitro* culture) by improving mass transport, therefore, cellular viability, and facilitating tissue maturation [216]. Crosslinking strategies after printing are typically used to stabilise either the printed hydrogel within the support bath or the bath itself, using thermal, ionic, enzymatic, or light-mediated mechanisms. Alginate has been extensively used for embedded bioprinting because its rheological properties can be tuned, for example, by partial pre-crosslinking or by adding thickening agents such as gelatin, which improves print fidelity and resolution [217]. Alginate can also be chemically modified to enhance its biocompatibility properties, for instance, by oxidation to increase degradability [218] or by conjugation with RGD peptides to promote cell adhesion [219, 220]. However, achieving high resolution often requires elevated extrusion pressures, which can impose substantial shear stress on encapsulated cells and compromise their viability. In contrast, alginate at relatively low concentrations has a very low viscosity and is better suited to embedded bioprinting, where the support bath mechanically stabilises the printed filaments. When alginate is printed into a support bath, the bath typically contains divalent cations such as calcium chloride or calcium sulphate, enabling immediate ionic crosslinking of the alginate upon extrusion and simplifying subsequent bath removal [221, 222]. Gelatin is widely used in bioprinting due to its high cytocompatibility, intrinsic cell-adhesive properties and enzymatic degradability, all of which support cell adhesion, spreading and growth [223]. Like alginate, gelatin is frequently modified to enable controlled crosslinking and stabilisation. Unmodified gelatin, derived from partial collagen degradation [224], undergoes thermoreversible gelation just below room temperature; it is shear-thinning and presents cell-adhesive ligands (including RGD sequences) that can be cleaved by cells via matrix metalloproteinases (MMPs) [225]. This makes gelatin an attractive partially or fully sacrificial bioink, as it can be remodelled by encapsulated cells and facilitates deposition of new ECM during tissue maturation. Due to the presence of reactive functional groups along its backbone, gelatin is also straightforward to chemically modify for the production of crosslinkable bioinks [226]. The most commonly used derivative is gelatin methacryloyl (GelMA), in which methacrylate groups are introduced, enabling the

formation of a dense, covalently crosslinked network under UV light exposure [227]. Previous studies have shown that tuning bioink degradability can enhance ECM deposition by encapsulated cells in cartilage TE. In contrast, increasing crosslinking density to obtain stiffer bioinks generally reduces degradability, can hinder the formation of new ECM and tune the phenotype of the bioprinted cells. For example, it has been demonstrated that tuning the stiffness of alginate-based bioinks can direct MSC fate, with softer formulations promoting adipogenic differentiation and stiffer formulations inducing osteogenic differentiation in the printed construct [228]. It is therefore essential to finely control hydrogel degradation when cells are encapsulated, so that the material design strikes an appropriate balance between structural stability and cell-mediated remodelling.

In TE, other strategies have been reported to create naturally inspired hydrogels by extracting and processing materials directly from native tissues. This is where engineering and biology intersect most clearly. In recent years, a wide range of naturally derived biomaterials has been developed, including protein-based systems such as silk fibroin, as well as ECM-derived materials and bioinks. ECM extracted from different tissues has been used to formulate tissue-specific bioinks, or in more traditional formats such as plate coatings (e.g. fibronectin), to better recapitulate the biochemical and biophysical composition of native ECM [229]. When properly processed, ECM can retain tissue-specific biophysical and biochemical cues that support cell survival, organisation, differentiation, and maintenance of phenotype [230]. For example, Shimomura *et al.* (2016) extracted ECM from the inner and outer regions of the meniscus and combined it with bone marrow-derived MSCs. The inner meniscal ECM promoted a more fibrocartilaginous phenotype, whereas the outer ECM supported a more fibroblastic phenotype, reinforcing the concept that the native tissue-derived ECM might provide region-specific cues that direct cell fate [231]. However, collagen-based materials, due to their relatively long degradation times and printing challenges, are not ideal candidates for bioprinting μ Ts.

Compared to printing single cells, μ T bioprinting imposes additional design constraints. The bioink must not only be printable, but its mechanical and adhesive properties must support fusion, alignment, and integration of the μ T without impairing their remodelling capacity.

Consequently, bioink formulations must be carefully tuned to balance structural fidelity with biological performance (Fig. 2.5).

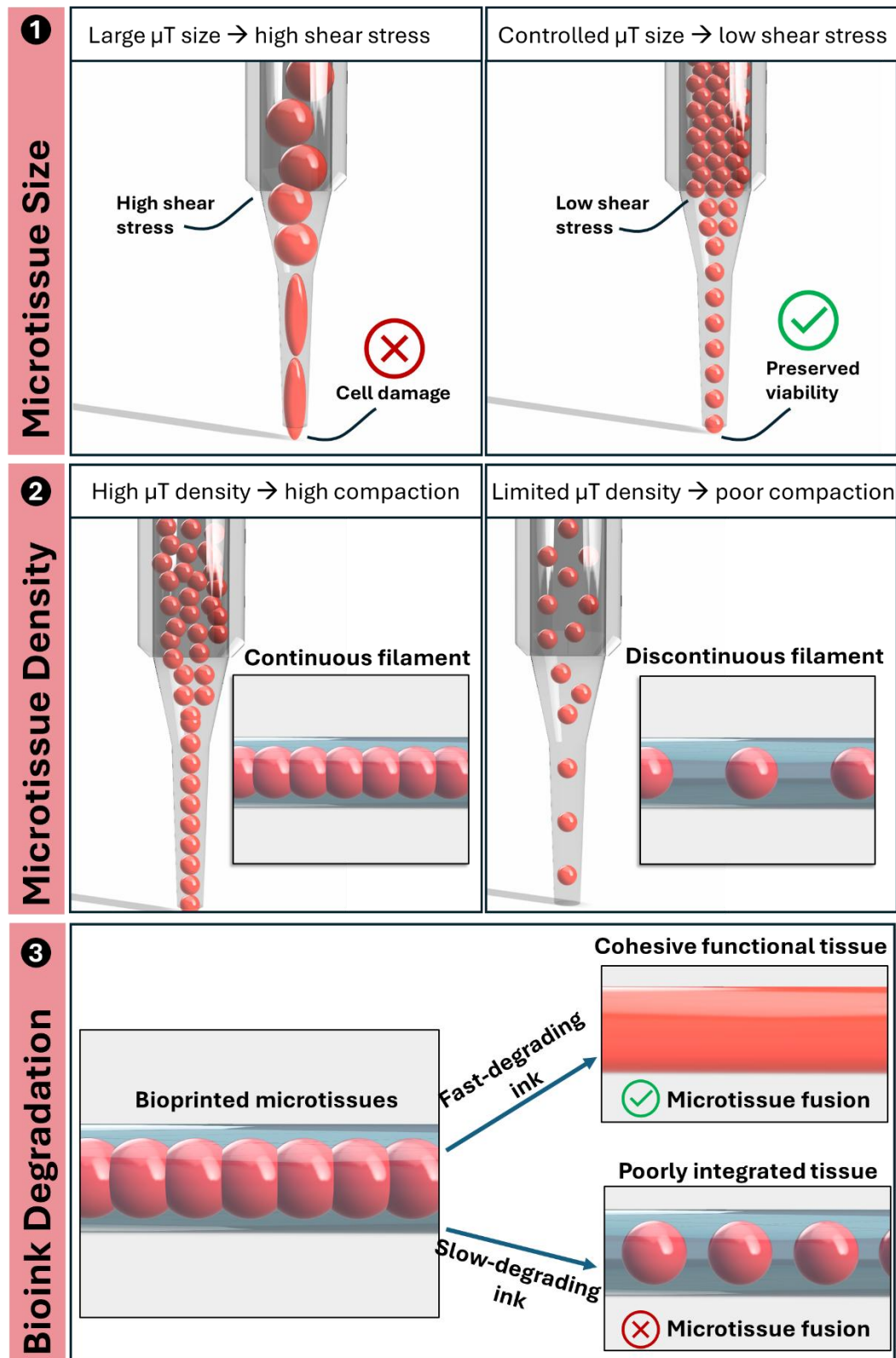


Figure 2.5. Schematic representation of key parameters for microtissue bioprinting. (1) Control of microtissue size reduces shear stress during extrusion, thereby preserving cell viability and internal organisation. (2) The density of microtissues within the cartridge before printing determines the extent of post-printing microtissue contact and fusion. (3) Bioink degradability governs microtissue fusion during maturation: a fast-

degrading or sacrificial ink promotes microtissue–microtissue contact, fusion, ECM remodelling and formation of a cohesive construct, whereas a slowly degrading bioink limits contact between microtissues, leading to maturation of individual microtissues but a non-cohesive overall construct. Figure created with Autodesk Fusion.

When printing μ Ts, several key parameters need to be considered: (1) μ T size must be controlled to limit shear stress during extrusion, thereby avoiding cell damage or disruption of internal organisation; (2) the density of μ Ts within the bioink must be sufficient to promote post-printing contact, compaction, and subsequent maturation, while maintaining reproducibility, geometric fidelity, and uniform tissue architecture; and (3) post-printing μ T fusion is the central event driving the formation of a cohesive, functional construct, so the bioink must be engineered to permit close cellular contact and active remodelling. These requirements come on top of the general criteria for embedded bioinks, including shear-thinning behaviour, rapid viscosity recovery after extrusion, and compatibility with post-printing crosslinking strategies. Sacrificial bioinks or rapidly degrading hydrogels are particularly attractive in this context, as their shear-thinning properties can facilitate printing of large cellular aggregates while enabling fusion after deposition [66].

- **Support bath**

Embedded bioprinting can greatly support the printing of μ Ts due to the stabilising nature of a support hydrogel when printing low viscosity inks required for μ T printing. To function effectively, the bath must display yield-stress behaviour: it should behave as a solid at rest to hold deposited filaments in place but locally fluidise under shear to allow nozzle movement and bioink deposition, then rapidly self-heal once shear is removed [200]. Common support bath formulations used in embedded printing are based on the same underlying principle: densely packed, jammed particulate systems that exhibit yield-stress behaviour [202, 204]. Differences between approaches arise primarily from the material composition and particle properties. FRESH is arguably the pioneering embedded printing approach, in which jammed gelatin microparticles form a yield-stress medium behaving like a Bingham plastic and thereby stabilising low-viscosity inks during deposition. Because the FRESH bath is gelatin-based, warming to 37 °C melts the matrix in line with gelatin's

thermoreversible gelation, enabling gentle recovery of the printed construct. Conversely, a non-sacrificial support bath can temporarily bear the mechanical load, preserving the construct shape that would otherwise rely solely on the bioink [232]. This can reduce the shear stress associated with extruding cellular aggregates (100-500 μm) that are orders of magnitude larger than single cells (10-30 μm). Simultaneously, the support bath can act as a guiding framework for the newly deposited ECM, encouraging alignment of the collagen network along predefined structures. Together, these features make embedded bioprinting a particularly powerful strategy for constructing biomimetic tissues from μTs or cellular aggregates (Table 1).

Table 1. Comparison of common embedded bioprinting support bath systems and their suitability for μ T bioprinting

Support bath	Support bath composition	Key advantages	Limitations	Relevance
FRESH (Freeform Reversible Embedding of Suspended Hydrogels)	Gelatin microparticle slurry (sacrificial)	Excellent print fidelity for low viscosity inks Construct retrieval via thermal melting	Fully sacrificial Limited long-term mechanical support Weak boundary guidance post printing	Poor retention of μ Ts after bath removal Limited control over fusion and alignment
SLAM (Suspended Layer Additive Manufacturing)	Jammed agarose microparticles (Partially sacrificial)	Tuneable yield-stress behaviour Construct retrieval via mechanical washing	Lacking controlled degradation Weak boundary guidance	Limited control over fusion and alignment
SWIFT (Sacrificial Writing into Functional Tissues)	Dense living cellular matrix composed of cellular aggregates (non-sacrificial)	Bioactive environment Supports vascularisation and integration	Limited geometric control Bath properties depend on cell type and behaviour	Promotes integration but reduces control over spatial organisation and anisotropy
XG-based support bath	Shear-thinning polysaccharide hydrogel (partially or fully sacrificial)	Good printability; biocompatible; tuneable viscosity; allows gradual degradation	Limited long-term structural stability; potential loss of boundary guidance over time	Supports μ T fusion but may compromise structural fidelity if degradation is too rapid
Methacrylate XG (XG-MA)	Composite hydrogel with tuneable degradability	Balances structural support and controlled degradation supports both alignment and fusion	Requires careful optimisation of ratios added system complexity Construct is hard to retrieve	Enables controlled μ T fusion while preserving anisotropy and improving mechanical integration

For μ T bioprinting, greater sacrificial behaviour and rapid degradability in the bioink promote μ T fusion and yield to a more cohesive tissue [66]. In contrast, combining a sacrificial bioink with a sacrificial support bath (i.e. FRESH, SLAM) is not advisable: once the bath is removed and culture medium is introduced, the absence of both bioink integrity and external support is likely to result in μ T dispersion into the medium rather than stable construct formation (Table 1). A suitable strategy to stabilise printed μ Ts while maintaining control over their fusion is to use a sacrificial μ T-laden bioink in combination with a non-sacrificial support bath. In this configuration, the bath's mechanical and rheological properties must be carefully balanced: stiff enough to preserve filament architecture [233], yet sufficiently permissive to permit extrusion and minimise shear-induced damage to μ Ts. Post-printing, the bath should enable μ T fusion by providing a permissive environment for tissue remodelling (Table 1). Bath attributes such as porosity, biocompatibility, and degradation kinetics will also influence nutrient and oxygen transport, waste removal, and long-term maturation. In platforms such as SWIFT (Sacrificial Writing Into Functional Tissue), the “living bath” is composed of densely packed living μ Ts that not only mechanically support the printed paths but also actively contribute to new tissue formation, illustrating how the support bath can shift from a passive stabiliser to an active participant in morphogenesis [205](Table 1). . However, this biologically active support bath reduces control over spatial patterning and anisotropic organisation, which are critical for musculoskeletal TE.

Therefore, an ideal support bath for μ T bioprinting must provide sufficient mechanical support to preserve printed architecture while enabling controlled degradation or remodelling to facilitate μ T fusion and tissue maturation. This requirement is not fully met by existing embedded bioprinting systems, motivating the development of alternative support strategies specifically tailored for μ T printing. In this context, xanthan gum (XG)-based support baths, particularly when modified or combined to tune their mechanical and degradation properties, offer a promising strategy to support μ T printing, guide tissue organisation, and enable controlled fusion and maturation (Table 1). Overall, the design of the support bath is a critical determinant of successful μ T bioprinting, requiring a finely tuned balance between mechanical guidance and permissiveness to enable both structural fidelity and functional tissue maturation.

- **Fusion of μ Ts**

Microtissue fusion is a fundamental process in bioprinting strategies, as successful fusion can transform initially distinct μ Ts into cohesive, functional tissue constructs [66, 170-172, 175, 212]. Several factors, including the maturation state of μ Ts, the composition of the bioink, spatial arrangement of the μ Ts, and post-printing microenvironment, influence an efficient fusion [234]. High packing density within the bioink and proper filament deposition patterns help maximise contact between adjacent μ Ts, promoting fusion via cytoskeletal remodelling and synthesis of new ECM (Fig. 2.5 B). A recent study by Decarli *et al.* (2023) examined extrusion bioprinting of MSC-derived μ Ts for cartilage regeneration using a xanthan gum–alginate bioink. The spheroids exhibited sGAG and collagen type II deposition, indicating that the ink and process preserved viability and supported chondrogenesis. However, the low spheroid loading did not produce a fully cohesive tissue; substantial hydrogel remained between spheroids, limiting fusion, preventing maturation toward the intrinsically anisotropic architecture of articular cartilage, and yielding very low mechanical properties (~ 5 kPa) [235], well below those of even immature cartilage [236]. Conversely, in a recent study by Scalzone *et al.* (2024), where substantial μ T fusion was achieved, dermal μ Ts were printed into a Pluronic support bath at a higher density leading to a more cohesive tissue.

However, the density of microtissues within the ink is not the only factor influencing post-printing fusion. The maturation state of the μ Ts, together with bioink-dependent parameters that influence ECM composition, also regulates the kinetics and overall quality of the engineered tissue [164]. The dynamics of hydrogel degradation are already recognised as critical in scaffold-free bioassembly strategies, but they become even more important in the context of μ T bioprinting. During printing, μ Ts are exposed to shear-induced stress, and the bioink present between them can act as a physical barrier that hinders close μ T– μ T contact. Kronemberger *et al.* (2024) highlighted the importance of rapidly degrading hydrogels for μ T printing, showing that fast-degrading hydrogels better support μ T fusion than more slowly degrading counterparts. In this study, early hydrogel

degradation enabled earlier post-printing fusion of μ Ts and resulted in more cohesive tissue formation compared with hydrogels that degraded more slowly [66]. Daly *et al.* (2021) introduced a bioprinting method that positions spheroids within self-healing hydrogels, enabling precise spheroid patterning without the use of a bioink to achieve superior μ T fusion. In this approach, spheroids are aspirated from the medium, translated through the gel, and deposited with rapid bath recovery, then fuse into prescribed tissues with defined geometry and heterogeneity. The platform is well-suited for scaffold-free, dense, heterogeneous *in vitro* disease models and drug screening; however, it appears less suitable for engineering highly anisotropic tissues, and the serial aspiration workflow is too slow at high μ T counts, prolonging print times and potentially compromising viability [65].

Although previous studies have begun to identify and optimise key bioprinting parameters for μ T printing, they have generally addressed these factors only individually or partially. A fully integrated platform that simultaneously considers μ T size and density, bioink rheology and degradability, support-bath properties, and post-print maturation has yet to be established. Developing such a platform will be essential to reliably generate cohesive, mature, and anisotropic tissues from bioprinted μ Ts.

- **Maturation post printing**

Following bioprinting, tissue maturation is a critical phase that determines the functional outcome of the engineered construct. In μ T-based bioprinting, maturation involves not only the fusion of adjacent spheroids but also the remodelling of newly secreted ECM, alignment of cells, and the development of tissue-specific phenotypes. This phase is highly dependent on the microenvironment provided both during and after printing. In μ T-based constructs, the initial ECM of the spheroids often needs to be remodelled to allow full integration into a continuous tissue [164]. This remodelling during and after fusion dictates the maturation of the μ T-based graft into a specific phenotype [237]. Oxygen diffusion further influences maturation post-printing; because oxygen diffusion lengths are on the order of 200 μ m, [238] much larger constructs require perfusion bioreactors, microchannel networks [206, 232], or pre-vascularized building blocks [41], otherwise, excessive hypoxia and waste accumulation impede maturation. Mechanical cues, such as substrate stiffness or dynamic

loading, play essential roles in guiding differentiation and functional maturation. Mechanical cues might accelerate progression toward function: cyclic compression or tensile strain promotes collagen organisation and increases modulus in tenogenic grafts [239, 240]. For musculoskeletal and cardiac tissues in particular, topographical alignment and/or electromechanical stimulation have been shown to enhance maturation, promoting myotube formation or synchronised contractions [241], respectively. Similarly, electromechanical pacing in cardiac models enhances sarcomere order, conduction velocity, and force [242, 243]. Topographical constraints are equally important: transient confinement, curvature, and fibre or filament orientation bias cell alignment and collagen deposition along prescribed axes [81]. The relevance of these constraints has also been demonstrated *in vitro*, where printed or patterned materials with cell-adhesive properties promote cell spreading and guide the alignment of newly deposited ECM [234, 244]. Cell spreading is accompanied by the formation of actin stress fibres, which generate anisotropic traction forces in elongated cells and induce lateral compressive forces, further reinforcing cell elongation and orientation [245, 246]. Researchers have already started looking into how to bioprint spheroids or μ Ts in ways that promote tissue anisotropy. Ahrens *et al.* (2022) developed a bioprinting strategy to program anisotropy in engineered myocardium using anisotropic organ building blocks (aOBBs). aOBBs are elongated μ Ts composed of hiPSC-derived cardiomyocytes that self-align, then are concentrated into a densely cellular bioink. During extrusion, aOBBs orient along the toolpath, enabling sheets patterned with linear or spiral printed filaments. The constructs display high cell density, increasing contractile force and conduction velocity over time, and outperform spheroid-based tissues [243]. The constructs are still limited in overall size and lack integrated vasculature; scaling and perfusion will be needed to move toward therapeutically relevant grafts. Collectively, these processes strongly influence graft maturation and steer the construct toward a more or less anisotropic tissue architecture. Beyond mechanical and boundary-condition cues, localised growth-factor presentation promotes *in situ* differentiation, tissue-specific ECM deposition, and phenotypic stabilisation, accelerating the transition from a printed construct to functional tissue [247]. In meniscus TE, co-delivery of CTGF and TGF- β 3 recruits endogenous progenitors and drives fibrocartilaginous differentiation, with controlled dose and release rate improving integration and mechanical outcomes [154]. For AC, stratified presentation of chondrogenic cues within printed

constructs supports zone-appropriate ECM assembly, stabilising a hyaline phenotype during long-term culture [248]. Tendon TE, similarly, benefits from spatial control of growth factors such as PDGF-BB, IGF-1, BMP-12/-7, and SDF-1 α to promote tenogenesis [249-252].

Overall, emerging materials, printing strategies, and DE concepts can be harnessed to deliver biochemical and/or biophysical cues that drive tissue maturation *in vitro*. Such features enable engineered bioprinted constructs to recapitulate not only gross geometry but also microarchitecture, including fibre orientation, zonal composition, thereby reinforcing stable phenotypic differentiation. By coordinating cue delivery between bath and ink, and by programming the kinetics of release, degradation, and mechanical conditioning, printed tissues can more faithfully reproduce native structural hierarchy and function during maturation.

2.6.2 Bioprinting of μ Ts along physical boundaries to direct neo-collagen formation

While bioprinting has made it possible to recapitulate both ECM composition and structure, fully reproducing the complex architectures required for physiological function remains challenging. A contributing factor is the widespread reliance on supporting hydrogels, which can restrict cell–cell interactions, limit matrix remodelling, and hinder the emergence of higher-order organisation needed for tissue maturation. To overcome these limitations, scaffold-free approaches such as cellular self-assembly and self-organisation have gained attention as promising strategies to engineer highly biomimetic tissues, allowing cells greater freedom to form native-like architectures through developmental-like processes [177, 178, 182, 183, 237]. These concepts mirror events during embryonic development, where growing tissues are mechanically confined by neighbouring structures that impose compressive loads and boundary constraints [181, 192, 253]. Such forces are transduced into biomechanical signals that guide tissue maturation, polarisation, and patterning. *In vitro*, these cues can be approximated through physical boundary conditions. Confinement, curvature, and oriented fibres or filaments can bias cell alignment and collagen deposition along prescribed axes [181]. Previous work has shown that when cell-laden collagen gels are mechanically constrained, fibroblast traction aligns collagen fibres along the permitted axis, forming strap-like, ligament-mimetic bands between anchors [254]. In practice,

uniaxial constraints of collagen gels between fixed posts create sustained tension, driving cell and fibril alignment along the line of constraint [255]. Similarly, constraining tissues in curved moulds imposes non-uniform stress fields that direct circumferential fibre orientation. Ring- or annulus-shaped boundaries, for example, generate hoop tension and promote meniscus-like circumferential alignment that better resists radial extrusion [256].

More recently, physical guiding structures have been engineered directly within constructs to advance tissue alignment and maturation. One approach uses anisotropic PCL “microchambers” printed as rigid frames, into which cells are deposited by droplet-based (inkjet) bioprinting. The combination of geometrically defined chambers and controlled cell seeding imposes uniaxial constraints that drive collagen organisation. After inkjet deposition, defined numbers of cells first self-aggregate into spheroids, then adhere to the PCL struts, and only subsequently elongate and align their cytoskeleton and newly synthesized collagen preferentially along the chamber’s major axis, as traction is transmitted to the rigid walls [43, 177, 257]. By strategically tuning chamber geometry (aspect ratio, channel width, and fibre spacing), tensile load paths are concentrated along the long axis, strengthening the boundary cue. This principle has been harnessed in PCL-based scaffolds for fibrocartilaginous and tendon-like tissues to steer circumferential or uniaxial collagen alignment during maturation [258]. Physical guiding structures can also be created without extrusion-based printing, for example, using optical modulation instability (OMI). In this strategy, a light beam propagating through a photoactive resin, splits into multiple self-focused beams that locally crosslink the resin into patterned microfilaments. These microfilament networks act as internal physical boundaries that direct the alignment of encapsulated cells, enabling the engineering of highly anisotropic tissues such as muscle [206]. The approach has also been applied to generate AC constructs with orthogonal collagen alignments (e.g. perpendicular and horizontal layers) [259]. However, scalability is currently constrained by light penetration depth in thicker tissues, and in some cases, the anisotropic properties achieved *in vitro* have not translated into improved regeneration when implanted *in vivo*.

With the advent of advanced 3D printing strategies, it is now possible to design “active” scaffolds or biomaterials that act as programmable boundaries, imposing controlled, time-varying constraints on developing tissues to direct collagen alignment [260]. In this context,

time effectively becomes a fourth design dimension. Four-dimensional (4D) bioprinted constructs are designed to undergo programmed, controllable morphological changes in response to physical, chemical, or biological stimuli [261]. The direction, magnitude, and timing of mechanical cues are encoded in the scaffold's geometry and material composition, as well as in its response to external stimuli that induce shape change and material [262]. Although this field is still emerging, particularly when integrating 4D materials with living cells, it holds considerable promise for developmental engineering. Shape-morphing materials could be coupled with cells or μ T inks to impose persistent uniaxial or circumferential tension, recapitulating aspects of *in vivo* mechanical environments such as vessel or muscle contraction [263]. In doing so, these systems could more effectively bias cell alignment and guide collagen deposition, enabling the generation of even more biomimetic, functionally anisotropic tissues.

Chapter III. The maturation state and density of human cartilage μ Ts influence their fusion and development into scaled-up grafts

The author of this thesis was the primary contributor to this chapter/published paper and was responsible for the design of the study, execution of all experimental work, data analysis, and preparation of the manuscript.

3.1 Introduction

Cartilage TE aims to partially or completely regenerate tissue that has had its function impaired by trauma or disease. Current clinical strategies, such as matrix-induced autologous chondrocyte implantation (MACI), fail to consistently promote the regeneration of functional hyaline cartilage [264-266] and is an unsuitable treatment modality for large scale joint degeneration. Therefore, there is an urgent need to develop scalable cartilage TE strategies capable of directing hyaline cartilage regeneration.

Limitations associated with the use of scaffolds for tissue regeneration have motivated increased interest in scaffold-free TE strategies [15]. Such approaches leverage the inherent ability of cells to self-organise in response to interactions with neighbouring cells and the extracellular matrix (ECM) [81]. Modular scaffold-free TE strategies often involve the use of numerous μ Ts, defined as highly cellular aggregates that secrete their own extracellular matrix, which are subsequently combined to biofabricate a more complex, macro-scale tissue [35, 267-269]. The goal of such approaches is that the final engineered graft will exhibit a structure, morphological composition and function that closely resembles the native tissue. μ Ts have found diverse applications in the field of tissue TE [270-272], including their use as biological building blocks in the biofabrication and 3D bioprinting of complex tissues and organs [64, 165, 269]. Despite their promise for cartilage TE, it remains unclear whether such modular scaffold-free approaches can be used to engineer scaled-up, functional AC grafts using clinically relevant cell sources.

Modulating the process and kinetics of μ T fusion and (re)modelling is integral to their use in TE [36, 273]. Successful fusion of multiple μ Ts involves the complete integration of the

cells and their extracellular matrix components to create a more cohesive tissue structure [164]. Fusion is known to be strongly dependent on the maturation levels of individual μ Ts (i.e., how long μ Ts are kept in culture in isolation before they are combined with other μ Ts), with superior fusion observed for less mature μ Ts. Bhumiratana *et al.* (2014) explored the kinetics of μ T fusion and how it can influence the final cartilaginous phenotype. They showed that μ Ts set tenascin boundaries after 7 days of growing independently before fusion. The presence of these boundaries could impair cohesive tissue fusion [164]. De Moor *et al.* (2020) observed fusion of cartilage μ Ts after they were independently matured for 7 days; however, the resulting tissue was not fully compact after a 21-day culture period [173]. Although previous studies have generally observed superior fusion using less mature μ Ts, the impact of such μ T maturation levels on extended tissue development post-fusion remains unclear. Therefore, in the context of cartilage TE, further work is required to identify the specific μ T maturation levels most suited to the development of scaled-up grafts. In addition, most existing strategies have focused on the fusion kinetics of relatively small numbers of individual μ Ts [170, 171, 274], providing limited insight into the cellular and molecular mechanisms that regulate the fusion process in larger engineered tissues.

The overall aim of this study was to assess the capacity of cartilage μ Ts generated from human bone marrow-derived MSCs to fuse and generate a functional, scaled-up cartilage graft. To this end, we first sought to investigate the kinetics of μ T fusion and subsequent chondrogenesis for μ Ts of differing maturation levels. During the initial stages of fusion, we assessed changes in cell-cell and cell-ECM interactions in less and more mature μ Ts. We then explored how μ T density would impact chondrogenesis in longer-term culture. We finally sought to engineer a scaled-up cartilaginous tissue (10 mm in diameter) by fusing a large number of immature μ Ts.

3.2 Materials and Methods

3.2.1 Isolation and expansion of hBM-MSCs

Human bone marrow mesenchymal stem cells (MSCs) were isolated from fresh human bone marrow samples obtained from 21-year-old female donors (Lonza, Switzerland). A total of 30 ml of fresh human bone marrow was collected under sterile conditions. High

Glucose Dulbecco's Modified Eagle's Medium GlutaMAX, (hgDMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin (Gibco), and human Fibroblast Growth Factor (FGF-2) at 5ng/mL, was used as the culture medium (termed 'XPAN' media). Cells were seeded at a density of 40-60 million cells per T-175 flask (Corning) in XPAN. The T-175 flasks were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 5% O₂. After 4 days of initial seeding, when the media was changed, 10 mL of the media volume containing non-adherent cells was collected and re-seeded in another T-175 flask to promote adherence of slower-adhering cells. The cells were cultured for 12-14 days under 5% CO₂ and 5% O₂, and the media was changed every 3 days. After 12-14 days, hBM-MSCs formed colonies, and the cells were trypsinised using 0.25% (v/v) Trypsin ethylenediaminetetraacetic acid (EDTA) and frozen at passage one. For all experiments, hBM-MSCs at passage 3 (P3) were used. All studies in this paper used human MSCs, except for the scaled-up grafts (section 3.4), where caprine MSCs were used.

3.2.2 Isolation and expansion of caprine BM-MSCs

Caprine bone marrow mesenchymal stem cells (MSCs) were isolated from the sternum of skeletally mature, female goats. The bone marrow was cut into 2mm³ pieces, vortexed for 2 minutes in XPAN to aid liberate cellular components and then seeded at a density of 5.7×10^4 cells/cm² and maintained in a humidified atmosphere under physioxenic conditions (5% CO₂, 5% O₂). After 72 hours, the flasks were washed twice with sterile PBS to remove non-adherent cells and debris. Cell culture media was exchanged for fresh XPAN every 2 days. When the MSC colonies were 80% confluent, cells were trypsinised as described in 2.1. For further expansion of cells, flasks were seeded at a density of 5×10^3 cells/cm² at 5% CO₂, 5% O₂ and expanded up P3 for all experiments.

3.2.3 Fabrication of MSC μ Ts

The μ Ts were fabricated as previously described [41, 275]. The 3D printed mould stamps containing either 401 or 1,889 micro resections and 4% (w/v) ultrapure agarose were sterilised in an autoclave at 120 °C for 20 minutes. Next, 6 mL of ultrapure agarose was gently poured into each well of the 6-well plates and allowed to cool down for 15 min after moulding it. Agarose moulds were then soaked overnight in Endothelial growth media (EGM), composed of Endothelial Cell Basal Medium-2 (EBM) (Lonza) supplemented with

MV Microvascular Endothelial Cell Growth Medium-2 BulletKit™ (Lonza), at 37 °C in a humidified atmosphere with 5 % CO₂ and 5 % O₂ to enhance cartilage formation [276]. MSCs were seeded in a density of $5,35 \times 10^5$ into the microwells in a final volume of cell suspension of 3 mL, to obtain 401 or 1889 μ Ts with 4,000 cells/ μ T. The MSCs were maintained in chondrogenic media (CDM) consisting of hgDMEM GlutaMAX supplemented with 100U/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 (all from Sigma), 2.5 μ g/mL amphotericin B, and 10 ng/mL of human transforming growth factor-beta 3 (TGF- β) (Peprotech, UK). 20 minutes after seeding, the plates were centrifuged for 5 minutes at 700 x g to collect cells at the bottom of each well. Due to the smaller area available for each μ T in the 1,889 micro resections mould system, cells were seeded at a density of $7,5 \times 10^5$ /mL into the microwells for a final volume of 5 mL of CDM and centrifuged as described above. Albeit the high density of cells in a low media amount, after 2 days the cells showed high viability (Fig. S3.1).

3.2.4 Fusion of μ Ts

To ensure that the exposure of the μ Ts in CDM was equal for all the groups, we cultured each group in CDM for a total of 28 days as follows. The ‘F-D2’ μ Ts were cultured in CDM for 2 days in their individual microwells before fusion, followed by a further 26 days of fusion in CDM. The ‘F-D4’ μ Ts were cultured in CDM for 4 days in their individual microwells before fusion, followed by a further 24 days of fusion in CDM. The ‘F-D7’ μ Ts were cultured in CDM for 7 days in their individual microwells before fusion, followed by a further 21 days of fusion in CDM. After each maturation time, the μ Ts were harvested, counted, and seeded onto 2% (w/v) agarose-coated wells in a 48-well plate. To harvest the μ Ts, the media was first flushed over the agarose surface using a micropipette and subsequently collected into a 50 mL tube. The agarose mould was then removed, inverted into a new 6-well plate, pre-filled with 3 mL of fresh media, and centrifuged at 750 g for 5 min to collect the μ Ts at the bottom of the well. The resulting μ T suspension was then strained through a pluriStrainer® (pluriSelect®, Leipzig, Germany) to retain any agarose fragments. The density of the μ T suspension was quantified, and the appropriate volume was used to seed a precise number of μ Ts into each construct. The number of μ Ts seeded into each well was 40 ± 3 to ensure

that each construct had the same size. Immediately after seeding the μ Ts, 2 mL of CDM was gently added into each well, and the plate was swirled to favour μ T fusion. The constructs were cultured for 21 days in CDM in physioxenic conditions, and the media was exchanged thrice a week. This experiment was performed twice with two different female human donors.

To obtain different constructs by varying the μ T density, day 2 μ Ts were harvested and fused as previously described into agarose-coated 24-well plates. Next, the number of μ Ts seeded for the fusion was respectively 50, 150 and 300 per construct. Immediately after seeding the μ Ts, CDM was gently poured into each well, and the plate was swirled to favour μ T fusion. The developing constructs were cultured for 42 days in CDM, in physioxenic conditions, and the media were exchanged thrice a week. The experiment was performed once with 4 replicates, with one female human donor.

3.2.5 Fabrication of scaled-up cartilage constructs using caprine MSCs

The μ Ts were fabricated according to the method described in 2.2 (using caprine as opposed to human MSCs) and cultured in CDM for 2 days in physioxenic conditions before harvesting. At day 2, μ Ts were harvested, counted, and seeded into non-adherent agarose wells. In this study, a cylindrical stamp with a diameter of 10 mm and a height of 1 mm was designed to cast the μ Ts. This stamp was used to create a positive mould under sterile conditions using a hot 2% (w/v) agarose solution. The 3D printed stamp was designed using Autodesk® Fusion 360™ software. 4,000 μ Ts were added to each agarose mould to fill the entire mould. This was done to ensure that the appropriate number of μ Ts was seeded to achieve the desired construct size and volume. After seeding the μ Ts, 80 mL of CDM was gently added, and the constructs were maintained at 20% O₂ for 42 days. The fused μ Ts were cultured under two distinct conditions: the static groups were cultured in a 150 mm x 25 mm Petri dish, while the dynamic groups were cultured in a spinner flask (Gibco, Fisher Scientific, UK) rotating at a speed of 110 rpm. Media was changed thrice weekly. The experiment was performed once, with 3 replicates in each group, with one donor.

3.2.6 Histological evaluation

Briefly, samples were fixed with 4% paraformaldehyde (PFA) solution overnight at 4°C. Following fixation, the samples underwent dehydration using a series of ethanol solutions

(from 50% to 100% v/v), followed by clearing in xylene and embedding in paraffin wax (Sigma). The samples were sliced into 5µm tissue sections and rehydrated. Haematoxylin and eosin (H&E) staining (Sigma) was used to assess the tissue morphology, the extracellular matrix, and the nuclei of the cells. 1% (w/v) solution of Alcian Blue (AB) 8GX in 0.1 M hydrochloric acid (HCl) was used to stain sGAG content and counter-stained with 0.1% (w/v) nuclear fast red to determine cellular distribution. A 0.1% (w/v) Picrosirius red (PSR) solution was used to visualise collagen deposition (all from Sigma). Stained sections were mounted using DPX solution and imaged using an Aperio ScanScope slide scanner (Leica, Germany). Immunohistochemistry was performed for collagen type I (Abcam ab90395 1:400), collagen type II (Santa Cruz sc52658 1:400), and collagen type X (Col X) (Abcam 49945, 1:200) as previously described [277].

Cell apoptosis within the core of the scaled-up graft was evaluated using TUNEL staining. In brief, slides were rehydrated following previously established protocols [278], then treated with proteinase K (20 µg/mL) (Sigma)) for 5 minutes at room temperature. After washing with 1X PBS, the slides were incubated with a DNA labelling solution (TdT Reaction Buffer, TdT Enzyme, Br-dUTP, deionised water) for 60 minutes at 37°C. Following several washing steps, an anti-BrdU-Red antibody was applied for 30 minutes at room temperature in the dark. The slides were washed again, and 7-AAD/RNase A staining buffer was applied as a counterstaining for 30 minutes at RT in the dark, in a humidified atmosphere. The slides were then mounted with gold antifade mounting medium (Sigma), and sealed using clear nail polish. Images were captured within 3 hours of staining using a Leica SP8 scanning confocal microscope. Representative images are presented as maximum projections of z-stacks.

3.2.7 Immunofluorescence evaluation

Briefly, after fixation in a 4% paraformaldehyde (PFA) solution overnight, the samples were resuspended in a 20% sucrose (w/v) (Sigma) solution. After 1 hour, the solution was replaced with a 30% (w/v) sucrose solution and allowed to incubate for 24 hours or until the sample settled to the bottom of the vial. The following day, the samples were embedded in OCT embedding matrix (VWR, IE) and flash-frozen using liquid nitrogen. The frozen samples were then stored at -80°C until slicing. The samples were sliced with a thickness of 20 µm. Prior to antigen retrieval, the slides were rehydrated using PBS 1X

(Gibco). Antigen retrieval was performed in a sodium citrate buffer made with 10mM Sodium Citrate, 0.05% Tween 20 (all from Sigma), pH 6, in a humid environment at 80 °C for 20 minutes. The slides were washed with PBS 1X and incubated with 10% (v/v) donkey serum (Sigma) for 30 minutes to allow the blocking of nonspecific sites. Samples were incubated with primary antibody in 2% BSA in a humidified chamber overnight at 4 °C. Samples were washed 3X with 1X PBS and incubated with a secondary antibody in 2% BSA for 1h at RT in the dark. Samples were washed 3X with PBS 1X and counterstained with a Hoechst staining solution 1 µg/mL (Sigma) for 15 minutes in the dark. Samples were washed 3X with PBS 1X and mounted with mounting media (Sigma). Prior to imaging, the slides were sealed with nail polish and stored in the dark at 4 °C. Immunofluorescence was performed for Vimentin (Abcam ab92547 1:200) to assess the cell-ECM interactions and Neural Cadherin (N-Cadherin) (Abcam ab98952 1:200) to visualise cell-cell interactions. Images were taken using a Leica SP8 scanning confocal microscope. Representative images are presented as maximum projections of z-stacks.

3.2.8 Live-dead assay

Cell viability was assessed using a live/dead assay kit (Invitrogen, Bioscience). The constructs were first rinsed in PBS 1X and then incubated for 1 hour in a solution containing 2 µM of calcein and 4 µM ethidium homodimer-1 (EthD-1). Following the incubation period, the constructs were rinsed again to remove any excess of the dyes and were imaged using a Leica SP8 scanning confocal microscope. Excitation wavelengths of 485 nm and 530 nm were used for calcein, while emission wavelengths of 530 nm and 645 nm were used for calcein and EthD-1. Maximum projection z-stack reconstructions were generated to analyse cell viability throughout the depth of the tissue, with the view from the top.

3.2.9 Biochemical analysis

For biochemical analysis, approximately 200 µTs were pooled per sample at each time point, and analysed for DNA, sGAG, and collagen content. Each sample represented one independent biological replicate (n = 3)

After retrieval, the samples were washed twice with 1X PBS and dried. Papain enzyme solution created with 3.88 U/mL of papain enzyme, 100 mM sodium phosphate buffer, 5 mM Na₂EDTA, and 10 mM L-cysteine at pH 6.5 (all from Sigma), was then added to digest

the samples at 60 °C for 18 hours. Immediately after digestion, DNA content was quantified using the Quant-iT™ PicoGreen® dsDNA Reagent and Kit (Molecular Probes, Biosciences). The amount of sulphated glycosaminoglycan (sGAG) was determined using a dimethylmethylen blue dye (DMMB, Sigma) binding assay. To exclude any background absorbance, the pH of the DMMB was adjusted to 1.5. A chondroitin sulphate standard (1 mg/mL, Sigma) was used, and the absorbance was measured at 530 and 590 nm using a Synergy HT multi-detection microplate reader (BioTek Instruments, Inc). The 530/590 absorbance ratio was used to generate the standard curve and determine the sGAG concentration from the digested sample. Total collagen content was determined by measuring the hydroxyproline content using a chloramine-T assay. The samples were mixed with 38% HCl (Sigma) (1:1 ratio) and incubated at 110 °C for 18 hours for hydrolysis. After cooling, samples were centrifuged at 5000 x g for 5 minutes, and let dry at 60 °C for 48 h. The sediment was reconstituted in 200 µL of ultra-pure H₂O. Chloramine T (2.82% w/v) and 4-(Dimethylamino) benzaldehyde (0.05% w/v) were added and incubated for 20 minutes at RT in the dark. The hydroxyproline content was quantified using a trans-4- Hydroxy-L-proline standard at a wavelength of 570 nm with a Synergy HT multi-detection microplate reader (BioTek Instruments, Inc). Hydroxyproline levels were estimated from the standard curve at a wavelength of 570 nm. The hydroxyproline-to-collagen ratio of 1:7.69 was used to calculate the collagen content.

3.2.10 RNA isolation and quantitative Real-Time PCR

At 24h and 7 days after fusion of the human MSC-derived µTs at different maturation levels (F-D2, F-D4, F-D7) the samples were washed 3 times with 1X PBS and then snap frozen for further processing. RNA was isolated from the samples using the Trizol method (Sigma-Aldrich), following the manufacturer's instructions. Chloroform extraction was performed, and the RNA was resuspended in RNase-free water. The RNA samples were then stored at -80°C. For cDNA synthesis, a HighCapacity RNA-to-cDNA™ Kit (Applied Biosystems™), was used to transcribe 500 ng of RNA from each sample into cDNA (20 µL) via polymerase chain reaction (PCR). Real-time PCR was performed using an Applied Biosystems instrument, and Taqman PCR universal master mix (ThermoFisher) was used for the PCR reaction. Human-specific TaqMan probes (ThermoFisher) were used for gene amplification. The qPCR amplification followed the following cycles: 50 °C for 2 minutes, 95 °C for 10 minutes and

40 cycles at 60 °C for 1 minute each. The levels of gene expression were quantified using the real-time PCR data and analysed with the $2^{(-\Delta\Delta Ct)}$ method. The expression levels were normalised to the average expression of the 18S rRNA housekeeping gene.

3.2.11 Diameter measurements and Fluorescence quantification

Diameter measurement of single μ Ts and growing engineered cartilage fused μ Ts, was conducted using a 4x objective of an inverted microscope (Primo Vert, Zeiss, USA), and calculated using ImageJ software (Wayne Rasband and Contributors, USA). Images were captured at hourly intervals until 8 hours post-fusion, daily until day 7, and weekly until week 3 post-fusion. Four independent measurements of each construct were obtained from each experimental replicate. For immunofluorescence quantification, the intensity of 3 specific regions of interests (ROI) within each sample was measured and quantified through ImageJ software. The intensity was then normalised by the number of nuclei in the ROI.

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, CA, USA). For diameter measurements, a two-way ANOVA was performed, followed by Tukey's multiple comparison test to assess the differences in diameter between groups over time. For biochemical analysis, a one-way ANOVA was performed followed by Tukey's multiple comparison test to assess the differences between groups. For qPCR analysis, a two-way ANOVA was performed, followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups. For Young's Modulus analysis an unpaired t-test with Welch's correction was performed to assess the differences between the two groups. Significance was accepted when $p < 0.05$.

3.2 Results

3.3.1 Human MSC-derived μ Ts secrete a cartilage-like extracellular matrix over time under chondrogenic culture conditions

The ability of human bone marrow-derived MSCs to undergo trilineage differentiation towards adipogenic, osteogenic and chondrogenic was assessed with the tripotentiality assay (Fig. S3.2). Afterwards, the ability of human MSCs to form μ Ts was assessed (Fig.

3.1A), and histological and biochemical evaluation of the single μ Ts were performed at different maturation levels (2, 4, 7 and 28 days) to quantify the level of chondrogenesis and extracellular matrix production (Fig. 3.1B, C). At days 2, 4, and 7 of culture, no strong indication of chondrogenesis was observed (Fig. 3.1B). However, at day 28, the μ Ts stained positively for GAGs and collagen deposition (Fig. 3.1B). The biochemical analysis showed a significant increase in collagen and sGAG production over time in culture (Fig. 3.1C). By day 28, there was a 2.5-fold increase in sGAG/ μ T ($p = 0.01$) and a 6-fold increase in collagen/ μ T compared to day 2 ($p = 0.0032$) (Fig. 3.1C). The sGAG/DNA ratio ($p=0.0108$) and collagen/DNA ratio ($p=0.0085$) also significantly increased with time in culture (Fig. 3.1C).

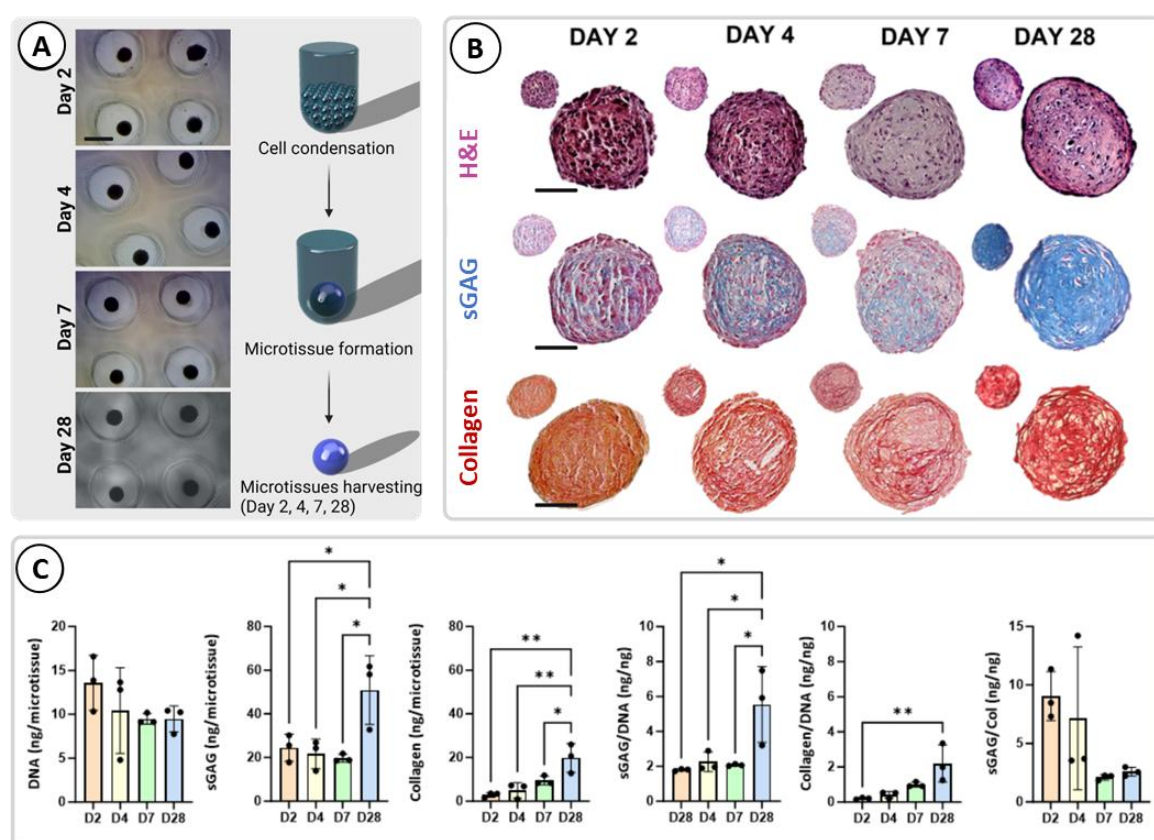


Figure 3.1. MSC-derived μ Ts secrete a cartilaginous matrix over 28 days of culture in chondrogenic media. (A) Experimental settings: μ Ts are cultured in agarose micro-moulds. (B) Histological panel for matrix components after 2, 4, 7 and 28 days in chondrogenic media. (C) Biochemical quantification of the μ Ts; levels of DNA, sGAG, and collagen are normalised to the number of μ Ts and to the DNA content. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, when tested using a one-way ANOVA ($n=3$) followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups. Scale bar is 60 μ m. Fusion 360™ was used to design the graphical abstract, and BioRender was used for the layout of the final figure.

3.3.2 Cell-cell and cell-ECM interactions during fusion depend on μ T maturity

To assess the capacity of μ Ts at different stages of maturation (day 2, day 4 and day 7) to form self-organised cartilaginous constructs by fusion, 40 μ Ts from each maturation group were combined and allowed to fuse (Fig. 3.2). The fusion of μ Ts was initially assessed using brightfield microscopy, capturing images at different time points to evaluate the fusion process over 21 days of chondrogenic induction (Fig. 3.2A).

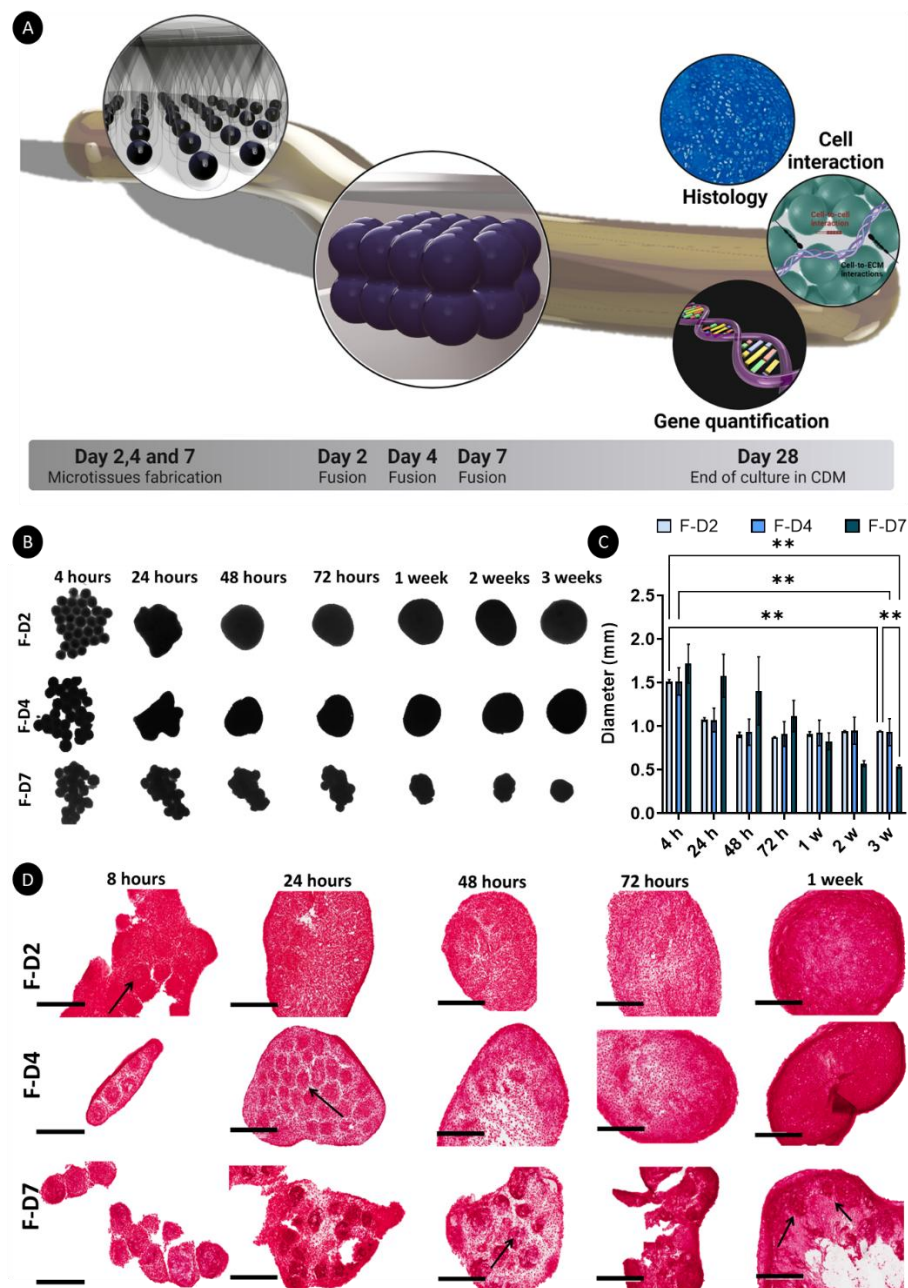


Figure 3.2. μ Ts of differing maturation levels show differences in fusion kinetics, final size and collagen deposition over 28 days of culture in chondrogenic media. A) Microtissues derived from human bone

marrow were cultured separately in agarose microwells for 2, 4, and 7 days (FD-2, FD-4, and FD-7, respectively). Following this individual culturing period, the microtissues were fused to create a larger tissue. All samples were assessed through histology, immunohistochemistry, biochemistry, and qPCR. B) Brightfield microscope images depict fused μ Ts observed over a 21-day period in culture in chondrogenic media, followed by C) diameter measurements. Scale bar: 500 μ m. D) Samples were subjected to picrosirius red staining to visualise the collagen present within the tissue. The arrows point to remnants of the structure of the individual μ Ts. Scale bar: 200 μ m. Significant differences are denoted as: ** $p < 0.01$, when tested using a two-way ANOVA test ($n=20$) followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups. F-D2: μ Ts fused at day 2 maturation level. F-D4: μ Ts fused at day 4 maturation level. F-D7: μ Ts fused at day 7 maturation level.

All μ Ts successfully fused in culture, although the fusion kinetics varied between the groups (Fig. 3.2A). For the day 2 maturation level (F-D2), the μ Ts fused into a compact tissue unit within 48 hours. For day 7 maturation level (F-D7), the μ Ts achieved compact fusion only after 7 days of culture (Fig. 3.2B). Additionally, the final size of the construct depended on the maturation levels of the individual μ Ts, with the F-D2 μ Ts producing larger (1 mm) constructs than the F-D7 (0.5 mm) μ Ts (Fig. 3.2A).

To investigate collagen deposition during μ T fusion, PR staining was used to monitor spatial changes in collagen accumulation for the different maturation levels (Fig. 3.2B). Clear collagen-rich boundaries were evident between the more mature (F-D4 and F-D7) μ Ts, especially at early timepoints (Figure 3.2C, black arrows). As time elapsed, the boundaries between the individual μ Ts were less obvious in day 2 μ Ts (F-D2) (Fig. 3.2B, black arrows). Evidence of residual boundaries was still clear after 7 days of fusion for the more mature μ Ts (Fig. 3.2B, black arrows).

We next examined the expression (qPCR) and spatial localisation (immunohistochemistry) of vimentin and N-cadherin, markers of cell-ECM and cell-cell interactions, respectively, at different timepoints post-fusion (Fig. 3.3).

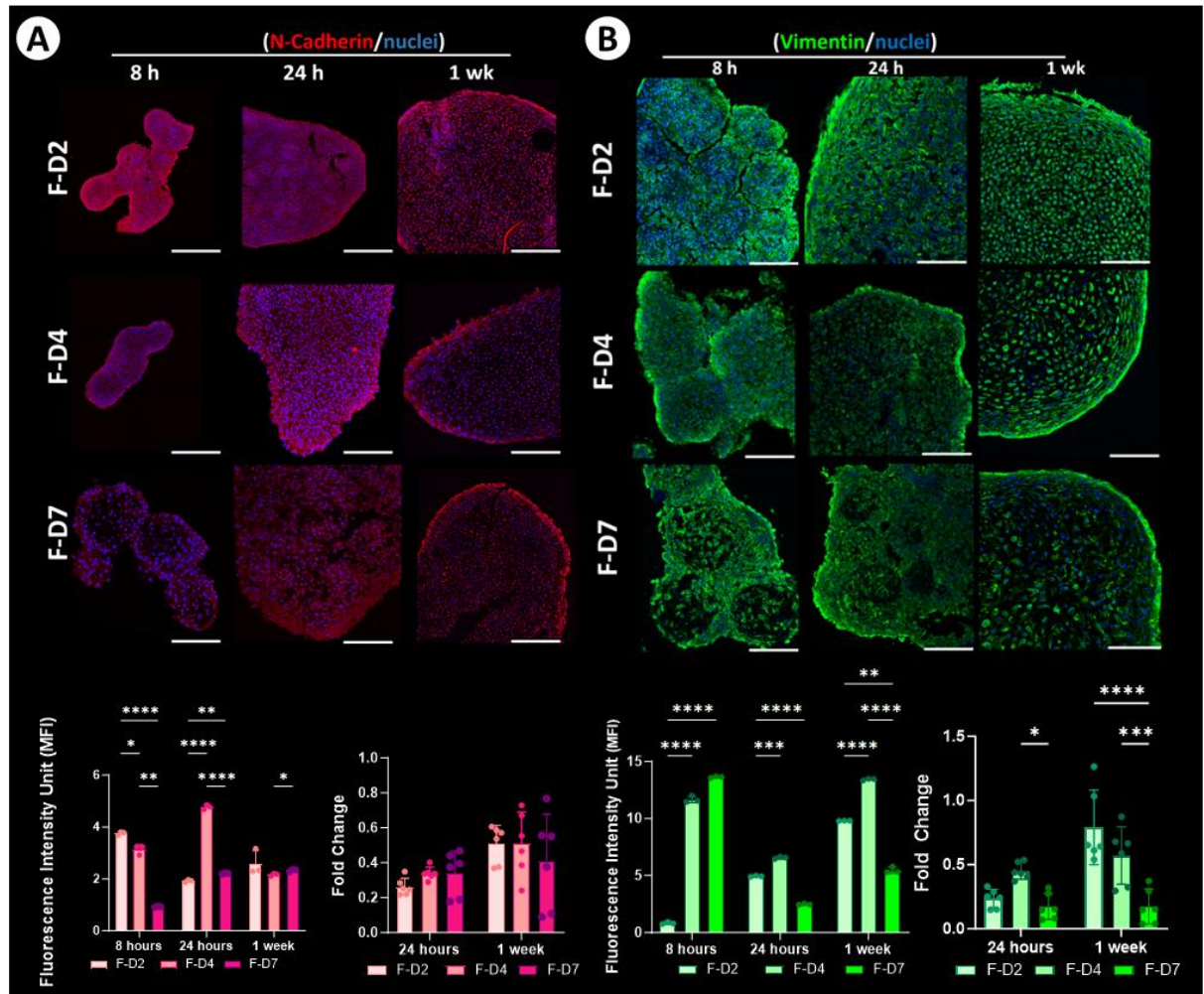


Figure 3.3. Cell-cell and cell-ECM interactions during μ T fusion are dependent on their maturation levels.

N-Cadherin and Vimentin are stained with respective monoclonal antibodies, and nuclei are stained with Hoechst staining. Immunofluorescence quantification was performed by normalising the region of interest (ROI) with the number of nuclei in the same ROI. mRNA expression of the cell-cell and cell-ECM markers, vimentin (VIM) and N-Cadherin (CDH2), were analysed by real-time PCR. Samples were normalised with MSCs in monolayer. A) N-Cadherin staining, immunofluorescence quantification (left graph) and mRNA expression (right graph) for fused μ Ts at different maturation levels over 7 days. B) Vimentin staining, immunofluorescence quantification (left graph) and mRNA expression (right graph) for fused μ Ts at different maturation levels over 7 days. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a two-way ANOVA test ($n = 6$) followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups. F-D2: μ Ts fused at day 2 maturation level. F-D4: μ Ts fused at day 4 maturation level. F-D7: μ Ts fused at day 7 maturation level. Scale bar: 200 μ m.

8 hours post fusion, more intensive staining for N-Cadherin was evident in the less mature F-D2 μ Ts compared to their more mature F-D4 and F-D7 μ Ts (Fig. 3.3A). Conversely, less intense vimentin staining was observed in the less mature F-D2 μ Ts at

this early timepoint (Fig. 3.3B). Vimentin staining increased over time during the fusion of the less mature F-D2 μ Ts, with an opposite trend observed in the more mature F-D7 μ Ts over time in culture (Fig. 3.3B). After 7 days of fusion, more homogeneous vimentin staining was observed in the F-D2 group compared to F-D7 group (white boxes, Fig. 3.3B). Gene expression quantification confirmed a significant increased vimentin expression at 7 days post-fusion within the less mature F-D2 μ Ts (Fig. 3.3B).

3.3.3 Fusion of less mature μ Ts promotes chondrogenic differentiation

We next examined the expression of a range of cartilage-specific genes 24 hours and 7 days post-fusion (Fig. 3.4). After 24 hours, the more mature F-D4 and F-D7 μ Ts expressed significantly higher levels of the cartilage-matrix genes ACAN and COL2A1 (Fig. 3.4). Notably, the expression of COL1A1 was highest in the mature F-D7 μ Ts at this early stage. 7 days after fusion, a significant increase in the expression of ACAN and COL2A1 was observed in the less mature F-D2 μ Ts. By this later time point, the expression levels of ACAN, COL2A1, and SOX9 were higher in the F-D2 μ Ts compared to the F-D7 μ Ts (Fig. 3.4).

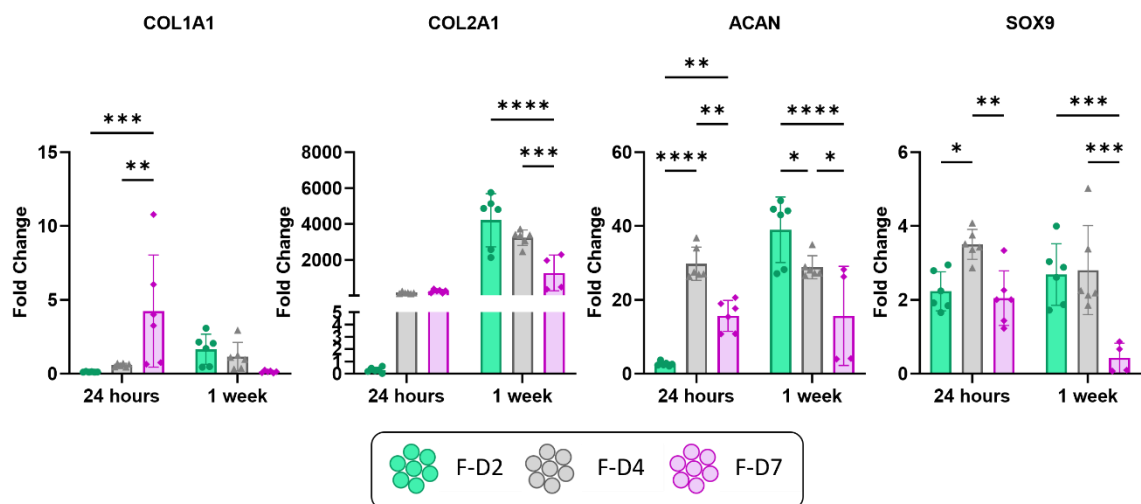


Figure 3.4. Chondrogenic gene expression following μ T fusion. mRNA expression of chondrogenic markers from left to right: COL1A1, COL2A1, ACAN and SOX9 analysed by real-time PCR and normalised against the housekeeping gene 18s rRNA. Each sample was normalised against the negative control of monolayer cells cultured for 2 days. F-D2: μ Ts fused at day 2. F-D4: μ Ts fused at day 4. F-D7: μ Ts fused at day 7. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 when tested using a two-way ANOVA test (n=6) followed by Tukey's multiple comparison test to assess the difference in the gene expression

between groups.

We next examined how μ T maturation levels could influence cartilage-specific ECM components after 28 days of chondrogenic induction (Fig. 3.5). As a control, we added the single μ Ts cultured for 28 days in isolation within microwells to assess if there was a change in the chondrogenic markers between fused and non-fused μ Ts.

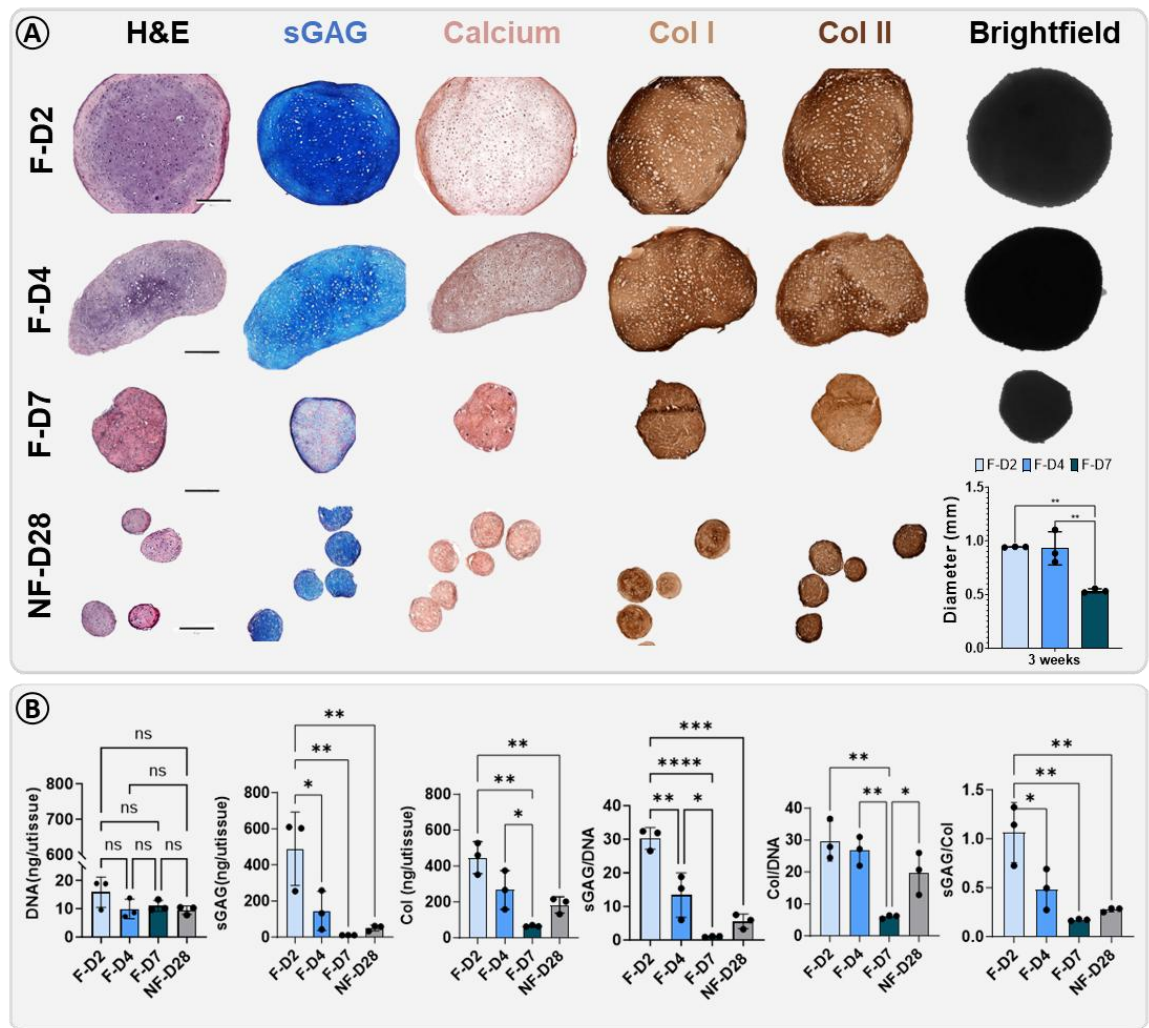


Figure 3.5. Fusion of μ Ts at day 2 supports cartilage tissue formation. A) Histological panel for standard chondrogenic markers: haematoxylin and eosin (H&E) staining, alcian blue staining, alizarin red staining, immunohistochemistry for collagen type I and collagen type II) after 28 days in chondrogenic media (scale bar: 250 μ m). Last column is a brightfield microscope image of fused μ Ts at 28 days in chondrogenic media. B) Biochemical quantification of the μ Ts, levels of sGAG and collagen normalised to DNA content. Significant differences are denoted as: * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 when tested using a one-way ANOVA test (n =3) for biochemical analysis and a two-way ANOVA for diameter measurements, followed by Tukey's multiple comparison test to assess the difference in the

gene expression between groups. F-D2: μ Ts fused at day 2 maturation level. F-D4: μ Ts fused at day 4 maturation level. F-D7: μ Ts fused at day 7 maturation level.

Positive staining for sGAGs (alcian blue) and Col II was observed in all groups, with the most intense staining for sGAGs observed in the less mature F-D2 group (Fig. 3.5A). The DNA level quantification shows no statistical changes between groups ($p=0.1686$). Biochemical analysis further confirmed significantly higher ($p<0.0001$) sGAG/DNA and collagen/DNA levels in the constructs generated with the less mature F-D2 μ Ts (Fig. 3.5B). Fused μ Ts (F-D2 and F-D4) exhibited a greater ability to produce sGAG ($p<0.001$) compared to individual μ Ts cultured for 28 days in the microwells, indicating differences between the fused and non-fused μ Ts (Fig. 3.5B). Due to their superior capacity for chondrogenesis, the F-D2 μ Ts were used for subsequent studies.

3.3.4 Increasing the density of μ Ts per construct facilitates the engineering of mechanically functional grafts of a clinically relevant size

We next sought to assess how the number of μ Ts used to generate a scaled-up construct would impact subsequent tissue development (Fig. 3.6). To this end 50, 150 or 300 μ Ts (F-D2) were fused and tissue morphology was assessed after 42 days of culture (Fig. 3.6).

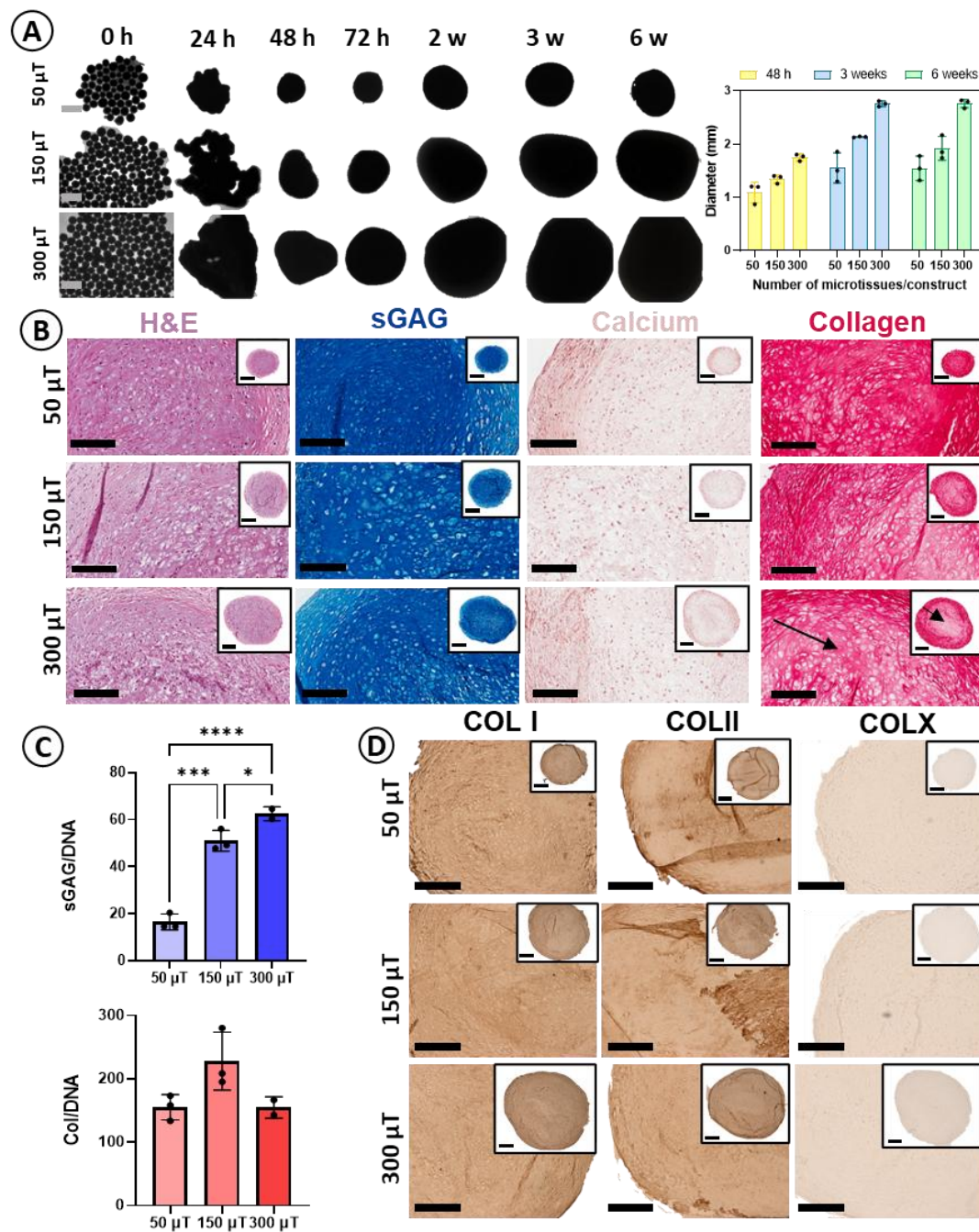


Figure 3.6. Increasing the density of μ Ts per fused construct improves chondrogenesis. A) brightfield imaging of fused μ Ts over a period of 42 days in culture in chondrogenic media followed by diameter measurement. Statistical differences were analysed using two-way ANOVA followed by Tukey's post hoc test; however, no significant differences were observed between groups at the same time point. Scale bar: 500 μ m. B) histological panel of fused μ Ts after 42 days in culture for standard chondrogenic markers: haematoxylin and eosin (H&E) staining, Alcian blue staining (AB), alizarin red staining (AR) and picrosirius red staining (PSR). Scale bars: 200 and 400 μ m. C) Biochemical quantification for chondrogenic markers (sGAG, Collagen). D) Immunohistochemistry for collagen types I, II and X. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a one-way ANOVA test ($n = 3$) followed by Tukey's multiple comparison test to assess the difference in the gene expression

between groups. μ T: μ Ts. Scale bars: 300 and 400 μ m.

The diameter of each construct increased over 42 days of chondrogenic induction, with no statistical differences among the groups (Fig. 3.6A). The relationship between the total input μ T volume and the resulting fused construct volume was evaluated by estimating volumes from μ T size and measured construct dimensions (Fig. S3.3). Based on an average μ T diameter of ~ 175 μ m, the estimated input volumes for 50, 150, and 300 μ Ts were ~ 0.14 , 0.42 , and 0.84 mm^3 , respectively, while measured construct diameters of ~ 1.8 mm and ~ 2.8 mm yielded corresponding output volumes of ~ 3.05 mm^3 and ~ 11.49 mm^3 , assuming spherical geometry (Fig. S3.3). Increasing the number of μ Ts per construct led to a significant increase ($p < 0.0001$) in sGAG production (Fig. 3.6C), while collagen synthesis appeared less dependent on μ T density (Fig. 3.6C). Collagen deposition appeared homogeneous in the 50 μ T group, while a core region staining less intensely for collagen appeared to develop in the 300 μ T group (Fig. 3.6A, arrow). sGAG deposition appeared equally homogenous in all the groups (Fig. 3.6B). After 42 days, a stable cartilage phenotype was achieved in all groups without signs of hypertrophy, as indicated by negative alizarin red and collagen X staining (Fig. 3.6B, D).

To create a larger construct of a clinically relevant size, a cylindrical stamp with a diameter of 10 mm and a height of 1 mm was designed to cast approximately 4,000 FD-2 μ Ts (generated using caprine MSCs). The constructs were cultured in dynamic (spinner flask) and static (Petri dish) conditions to assess how such culture conditions would impact tissue development and biomechanical function (Fig. 3.7).

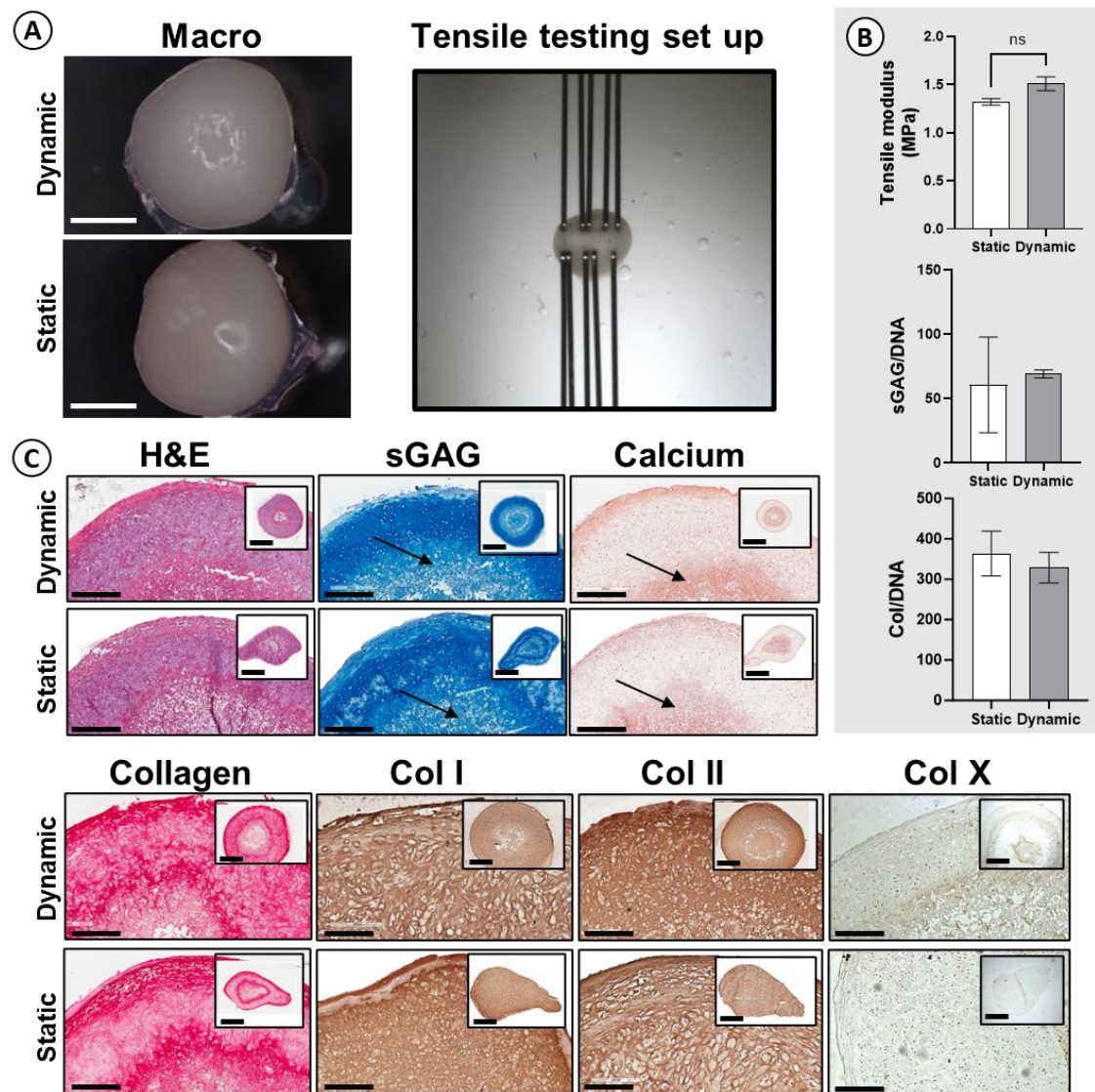


Figure 3.7. Engineering of scaled-up grafts using cartilage μ Ts. A) Stereomicroscopy (macro) images of a scaled-up engineered construct after 42 days of chondrogenic induction (scale bar: 2 mm); set up for tensile testing of fused μ Ts after 42 days in culture using a tensile biaxial tester (CellScale). B) Tensile modulus quantification of fused μ Ts after 42 days in culture in CDM. Biochemical quantification of constructs after 42 days of culture. Statistical analysis using a t-test revealed no significant differences between groups ($p > 0.05$). C) Histological and immunohistochemical panel of chondrogenesis markers: H&E, Alcian Blue, Alizarin Red, Picrosirius Red, collagen 1 (COL I), collagen 2 (COL II), collagen 10 (COL X). Scale bar: 400 μ m and 2 mm.

Apoptosis staining (TUNEL) at the end of the cell culture suggests little or no cellular apoptosis in both groups (Fig. S3.4). Histological analysis showed positive staining for sGAG in both static and dynamic culture conditions, with less intense staining observed in the centre of the graft (Fig. 3.7B, arrow). The formation of this 'core' occurred at

approximately 1 mm into the static constructs and 1.3-1.4 mm into the dynamic constructs (Fig. 3.7B, arrow). The AR staining suggested some calcium deposition in the centre of both groups (Fig. 3.7, arrow). The biochemical analysis showed no significant difference between the groups for sGAG and collagen deposition (Fig. 3.7 B). Uniaxial tensile testing was performed to failure to evaluate the mechanical properties of the constructs. Samples of defined geometry were tested at a constant strain rate, and stress–strain curves were analysed using engineering stress. The tensile modulus was comparable between constructs cultured under static and dynamic conditions, with no significant differences observed ($p > 0.05$). In addition, both groups stained positive for COL I and COL II (Fig. 3.7), suggesting the development of a more fibrocartilaginous tissue.

3.4 Discussion

In this study, we explored the fusion of μ Ts at varying stages of maturation to engineer scaled-up cartilage grafts. Our findings indicate that fusing μ Ts at lower maturity levels promotes enhanced chondrogenesis in the resultant grafts. Moreover, we investigated the role of cell-cell and cell-ECM interactions during μ T fusion by assessing temporal changes in N-cadherin and vimentin expression and localisation during the initial stages of fusion. We found that increased N-cadherin expression in less mature μ Ts was associated with stronger cellular interactions, as evidenced by accelerated and more robust fusion. We next explored how μ T density impacted subsequent chondrogenesis, observing that sGAG synthesis increased with increases in μ T number. We finally explored the scaling-up of the tissue-engineered graft by dramatically increasing the number of fusing μ Ts. Our results demonstrate that increasing the number of μ Ts does not hinder fusion, validating the feasibility of using human MSC-derived μ Ts for the engineering of cartilaginous constructs of a clinically relevant size.

We initially evaluated the composition of individual cartilage μ Ts after 2, 4, 7 and 28 days of culture within single microwells. Despite there being few changes in ECM deposition during the first 7 days of μ T maturation, the capacity of these cellular structures to fuse did noticeably change during this first week of culture. Previous studies have explored the fusion of μ Ts [164, 171, 173, 273], demonstrating that

increasing μ T maturation periods per-fusion negatively impacts the subsequent fusion process [164, 175], although the underlying mechanisms responsible for this remain unclear. In our study, we investigated fusion for μ Ts at early, intermediate, and later stages of maturation (day 2, 4, and 7, respectively). We observed that μ Ts fused at day 2 (F-D2) compacted much faster compared to more mature μ Ts (F-D4 and F-D7). This superior fusion was also associated with enhanced chondrogenesis at the end of a 28-day culture period, as evident by higher levels of sGAG and collagen deposition. These results point to the importance of ensuring robust and early fusion for supporting long-term cartilage tissue development.

As outlined in our study and corroborated by previous research [175], the presence of ECM plays a significant role in μ T fusion. Single μ Ts were typically characterised by the presence of circumferentially aligned collagen fibres. These collagen fibres became more evident with increasing μ T maturation. Interestingly, when μ Ts fused earlier, greater reorganisation of the collagen fibre network was observed. In the F-D7 group, intense collagen staining persisted around the individual μ Ts. These findings suggest that the spatial arrangement of collagen prior to fusion may influence the ultimate tissue architecture, μ T fusion, and consequently, the mechanical properties of the resulting graft [164, 172].

N-cadherin and vimentin are proteins intricately involved in cartilage development. N-cadherin is a transmembrane protein which functions to mediate cell-cell interactions (it is commonly used as a marker of cell-cell interactions) and is highly expressed during chondrogenesis [279-282]. In this study, we observed that accelerated fusion of less mature μ Ts was associated with greater N-cadherin expression in the early stages of fusion. Vimentin is a structural protein that plays a key role in cell-ECM interactions; it regulates cell adhesion strength by contributing to the development of complexes that control the formation of focal adhesion structures connecting cells to the matrix [283-286]. Vimentin staining has previously been used as a marker to assess fusion among neighbouring spheroids [65]. In our study, no discernible differences were observed in vimentin expression 8 hours after fusion across the different maturation groups. However, at 7 days post-fusion, the F-D2 group exhibited notably stronger positive staining for vimentin, while the F-D7 group displayed weaker staining. This suggests that successful fusion is associated with increased vimentin localisation around cells at later

timepoints as they mature and form a more developed cartilage matrix.

After investigating the mechanisms involved in early fusion, we investigated the ultimate tissue phenotype. Nilsson *et al.* (2021) demonstrated that the final tissue exhibits less intense sGAG staining when μ Ts are fused after 21 days compared to μ Ts fused after 1 day. Additionally, the overall biofabricated tissue appears more compact with higher sGAG intensity and more homogeneous collagen distribution [172]. Our study aligns with these previous findings, despite exploring a narrower range of μ T maturation levels (between day 2 and day 7). Specifically, the F-D2 group showed more intense staining for sGAGs compared to the F-D7 group, which exhibited a smaller final size. To confirm the change in the tissue type, we next explored changes in gene expression during fusion for μ Ts at different stages of maturation. As expected, the expression of chondrogenic genes was generally higher in the more mature F-D4 and F-D7 μ Ts after 24 hours of fusion. By 7 days of culture, ACAN and COL2 expression were significantly higher in the less mature F-D2 μ Ts, which suggests the development of a more cartilage-like phenotype in this group with time in culture.

The less mature F-D2 μ Ts were then employed to engineer larger grafts by increasing the density of μ Ts per tissue construct. Previous studies [64, 287] have explored μ T fusion by varying the number of cells per μ T while keeping the number of μ Ts constant. These studies revealed that the number of cells per μ T directly impacts the final graft cellular homogeneity by impacting the kinetics of fusion and the final tissue morphology. In our study, we investigated μ T fusion by modifying the μ T density per final construct, resulting in an increase in overall tissue size and volume. A recent study by Kopinski-Grünwald *et al.* (2024) [288] attempted to engineer larger tissues using spheroids as building blocks with a scaffold. While the μ Ts fused, the individual μ Ts were still discernible after 28 days in culture. In our study, the μ Ts in all groups compacted cohesively, and the tissue exhibited an increase in diameter and output volume, which might be related to an increase in GAGs production, which was prominent across all groups. Interestingly, no obvious signs of hypertrophy were observed after 42 days of culture in the presence of TGF β 3, suggesting the development of stable hyaline cartilage. A number of factors may explain the absence of hypertrophy within such μ T-based grafts. The high cell density within these engineered tissues likely leads to the development of a low oxygen microenvironment, which is known to suppress

hypertrophy of chondrogenically primed MSCs [289-291]. It has also previously been shown that extended chondrogenic induction *in vitro* allows MSCs to form stable cartilage tissue *in vivo* [292]. Therefore, the high levels of cell-cell interactions and low oxygen conditions within cell-rich μ Ts, coupled with extended chondrogenic induction *in vitro*, may allow for the development of stable hyaline cartilage. Further *in vivo* studies would obviously be required to evaluate whether our proposed approach can be used to generate phenotypically stable cartilage grafts resistant to calcification and endochondral ossification.

Our final goal was to fabricate a functional cartilage graft with a size comparable to a human clinical defect. After 42 days of *in vitro* culture, the tissues macroscopically appeared similar to cartilage, characterised by a white and translucent colour. Histological analysis of the tissue revealed positive staining for all chondrogenic markers... Nutrient transfer limitations within large, engineered tissues are widely recognised as a key challenge that can hinder matrix deposition [278, 293, 294], particularly in cell-dense constructs such as μ T-based constructs with high metabolic demands. In our study, when the μ T density was increased up to 300 μ Ts per construct, we observed changes in the histomorphology of the graft. While there was no significant change in GAG deposition, collagen deposition was notably impacted, with higher deposition observed in the annulus of the construct compared to the core. When culturing larger tissues, we sought to address this challenge by culturing the grafts under higher oxygen levels (20% O₂), which may have improved oxygen transport within the tissue. However, in tissue engineering, the size of the construct is not the sole consideration. Cell density within the graft is another crucial factor. Increasing the cellularity of MSC-based cartilage grafts has been shown to reduce oxygen levels within the core of the construct, which correlated with increased sGAG but reduced collagen accumulation in the centre of the engineered tissue [295]. Given that native articular cartilage exhibits a zonal architecture with spatial gradients in biochemical content, introducing appropriate gradients in oxygen, glucose, and other regulatory factors [296] may actually support the engineering of more biomimetic, scaled-up cartilage tissues. However, additional research is needed to identify conditions that could further enhance nutrient and oxygen transport in a quantifiable and controllable manner when engineering using cell-rich μ Ts and organoids.

One potential approach to overcome nutrient transport challenges is the utilisation of perfusion bioreactors [297-299], or the addition of rapidly degradable supporting gels [218, 300], which could aid in the transport of oxygen and nutrients within the self-organised tissue construct. In our study, cartilage constructs were cultured under static conditions (in petri dishes) and dynamic conditions (in a stirring flask) using media that was refreshed three times per week. No dramatic improvements in tissue development were observed with the introduction of dynamic culture, which may possibly be linked to the limited perfusion of nutrients through the graft in this particular culture set-up. However, this observation is limited by the small sample size ($n = 3$) and the specific culture set-up, which may have restricted nutrient perfusion within the graft. Perfusion bioreactors have previously been used to support the development of scaffold-free tissue-engineered cartilage [301], with the delayed application of perfusion found to be optimal as it gave time for cells to self-aggregate and consolidate prior to its application. A similar 'delayed' approach to perfusion is worthy of consideration for μ T-based grafts. Despite concerns associated with nutrient transport in our system, the resulting grafts were mechanically robust, with a tensile modulus approaching 1.5 MPa, albeit falling short of the reported values for AC, which can exceed 100 MPa for human cartilage [302]. These limitations are attributed to insufficient collagen deposition, crosslinking, and organisation. Therefore, it is imperative to explore additional strategies to improve the final ECM organisation to achieve a more mechanically robust cartilage construct when using a high density of μ Ts.

3.5 Conclusion

In this work, we successfully showed the potential of utilising μ Ts derived from human MSCs as biological building blocks to engineer large cartilage grafts. Our study demonstrated that promoting an earlier fusion led to a superior chondrogenic phenotype. Overall, these findings suggest that by carefully controlling μ T maturation levels and density that it is possible to engineer scaled up and mechanically competent cartilage grafts. However, a challenge that still needs to be addressed is the fabrication of hierarchical, organised and functional cartilaginous constructs. For this purpose, additive manufacturing (AM) techniques such as 3D bioprinting can offer a solution by

allowing precise spatial patterning of μ Ts layer by layer [183, 243]. Furthermore, 3D bioprinting strategies can potentially support the development of tissues within specific ECM organisation, which is essential to cartilage biomechanical function [303]. Therefore, this thesis will next focus on developing 3D bioprinting strategies to spatially pattern early maturation stage μ Ts to enable the engineering of scaled-up tissues.

Chapter IV: Toward a μ T-Based Bioprinting Platform for Musculoskeletal Tissue Engineering

4.1 Introduction

Building upon the work presented in Chapter III, where I optimised the timing of μ T fusion, the focus of this thesis now shifts toward translating those insights into a novel biofabrication platform. In Chapter III, it was demonstrated that early fusion of mesenchymal stromal cell MSC-derived μ Ts leads to more homogeneous and compact tissue constructs. This finding is crucial for advancing TE approaches, where the composition and structure of the engineered tissues are crucial. This chapter aims to leverage those outcomes from a biofabrication perspective—specifically, to establish and benchmark the critical steps required to spatially assemble μ Ts into predefined geometries suitable for engineering musculoskeletal tissues. The goal is to create a versatile bioprinting platform that incorporates the fusion dynamics of μ Ts as a core design parameter.

Bioprinting has emerged as a transformative technology in TE, offering precise spatial control over cell placement and the potential to fabricate complex, functional tissue constructs [304, 305]. While traditional bioprinting approaches have focused largely on the deposition of encapsulated single cells [43, 45], recent efforts have increasingly turned toward the use of μ Ts—multicellular aggregates that better mimic native tissue microarchitecture and cellular interactions[65, 177]. μ Ts provide several advantages, including enhanced cell–cell communication, improved viability, and a more physiologically relevant ECM profile [161, 306]. A major advantage of μ T-based biofabrication platforms is that they serve as ideal building blocks for engineering patient- and organ-specific tissues with defined cellular density, composition, microarchitecture, and function—ultimately contributing to biomechanically functional grafts [237, 288, 307].

However, the transition from single-cell printing to μ T-based approaches introduces new challenges, particularly in maintaining print fidelity, promoting timely and complete fusion between the μ Ts, and supporting cell differentiation during and after printing. Despite growing interest, comprehensive guidelines for designing and optimising bioprinting workflows for μ Ts are still lacking. Key variables—such as the choice of cell type, μ T

maturation state, bioink or hydrogel compatibility, and mechanical properties of the support bath—all significantly influence the final outcome. There is a clear need for defined criteria and experimental standards to guide decisions about when and how to print μ Ts effectively, especially in contexts demanding both bioprinting precision and biological functionality.

μ Ts have been extensively used across multiple fields, including disease modelling, drug testing, and regenerative medicine, particularly in the engineering of scalable musculoskeletal tissues. For instance, the Kenzan method was one of the first automated platforms developed to spatially organise μ Ts into predefined shapes [176, 308]. In this approach, spheroids are skewered onto a needle array using a robotic suction nozzle and then allowed to fuse in culture. This approach has been applied to the development of cartilage grafts, and although the cells remain viable and form a type I collagen-rich matrix, the resultant tissues show weak proteoglycan staining and offer limited structural improvement when implanted into cartilage defects. Similarly, Daly *et al.* (2019) developed a bioprinting approach in which μ Ts are transferred into a self-healing hydrogel support bath [65]. This enables the spheroids to fuse and form an engineered construct with geometry dictated by the printing process. Despite these advancements, such methods face limitations in producing complex, freeform 3D structures, especially along the z-axis, where layered curvature is required—making them less suitable for engineering intricate musculoskeletal geometries. Moreover, aspiration-assisted bioprinting is constrained by μ T size, as aggregates smaller than 500 μ m cannot be aspirated, limiting their use in high-resolution applications [309, 310].

In this context, embedded extrusion bioprinting presents a promising alternative. This technique enables the deposition of μ Ts of various sizes with minimal impact on cell viability when printed under low shear stress, while allowing for the fabrication of more complex 3D geometries. Embedded bioprinting involves extruding bioinks into a supportive matrix (such as a hydrogel), which stabilises low-viscosity materials during the printing process [311–313]. One notable example is the FRESH method, which uses a gelatin microparticle slurry to support the printed ink [314, 315]. The support bath exhibits Bingham plastic behaviour—remaining solid-like at low shear but flowing under higher shear stress—allowing the printing needle to move with minimal resistance while stabilising the extruded

bioink in its intended geometry. This technique has since inspired various other support bath formulations, including agarose-based (SLAM) [204], Pluronic-based [203, 316], and xanthan gum-based systems [211, 232, 235].

For successful μ T bioprinting, the surrounding bath must support both bioprinting fidelity and post-printing μ T fusion. Kronemberger *et al.* (2024) demonstrated that embedding μ Ts in oxidised alginate—which degrades within approximately seven days—facilitates fusion and formation of compact tissue constructs post printing [66]. In contrast, slower-degrading hydrogels like conventional alginate hinder this process. Furthermore, ensuring direct contact between adjacent μ Ts post-printing is essential for fusion but remains technically challenging. Fast degrading [218] or sacrificial bioinks offer a potential solution, enabling proper μ T placement while supporting rapid fusion post-printing. μ T-based printing also introduces unique technical challenges not encountered in cell-based bioprinting. These include μ T sedimentation in the cartridge over long print durations, nozzle diameter constraints, and the influence of μ T concentration on ink printability and filament continuity. Each of these parameters—alongside fusion kinetics—must be optimised to enable successful μ T printing.

This chapter aims to bridge that knowledge gap by outlining the critical parameters and practical considerations necessary for effective μ T bioprinting. Drawing from both the literature and our own experimental findings, a framework is proposed to guide material selection, process optimisation, and outcome evaluation in terms of fusion efficiency, cell differentiation, phenotype maintenance, and scalability. By integrating both engineering and biological perspectives, this framework establishes a foundation for standardised μ T bioprinting strategies, ultimately accelerating their use in regenerative medicine applications.

4.2 Materials and methods

4.2.1 Isolation and expansion of goat bone marrow-derived MSCs and μ T formation

Caprine bone marrow MSCs were isolated from the sternal marrow of skeletally mature females (see Chapter III). Marrow fragments ($\sim 2 \text{ mm}^3$) were vortexed for 2 min in XPAN, plated at 5.7×10^4 cells/cm², and cultured under physioxia (5% O₂, 5% CO₂). After 72 h, nonadherent cells were removed by two PBS washes; XPAN was refreshed every 2 days. At $\sim 80\%$ confluence, cells were trypsinised (Section 2.1) and re-seeded at 5×10^3 cells/cm² for expansion to passage 3.

The μ Ts were fabricated as previously described [275]. The 3D printed mould stamps (1,889 microwells) were used to mould 4% (w/v) ultrapure agarose, sterilised by autoclaving (120 °C, 20 min), cast into 6-well plates (6 mL/well), cooled 15 min, and soaked overnight in XPAN at 37 °C under physioxia [23]. MSCs were seeded into the microwells at 7.55×10^5 cells in 5 mL to target 1,889 μ Ts ($\sim 2,000$ cells each). Cultures were maintained in chondrogenic medium (hgDMEM GlutaMAX supplemented with 100 U/mL penicillin, 100 μ g/mL of streptomycin, 100 μ g/mL of sodium pyruvate, 40 μ g/mL of L-proline, 50 μ g/mL of ascorbate-2-phosphate, 4.7 μ g/mL of linoleic acid, 1.5 mg/mL of bovine serum albumin, 1X ITS, 100 nM dexamethasone, and 10 ng/mL TGF- β 3). Twenty minutes after seeding, plates were centrifuged at 700 g for 5 min to collect cells into the microwells and the plates were moved into a humidified atmosphere under physioxia conditions. Two days after seeding, the μ Ts formed, and they were harvested for bioprinting.

4.2.2 Preparation of gelatin and methylcellulose bioinks and bioprinting of μ Ts

Sterile gelatin type B (Sigma) at different weight percentages (1, 2, 3, 5 % w/v) was dissolved in sterile hgDMEM (Gibco) for 30 minutes at 37 °C in a water bath the day prior to printing. After dissolving, the tube with gelatin is removed from the water bath and placed at 4 °C overnight for gelation. Sterile methylcellulose (Sigma) was dissolved in sterile hgDMEM and left in a tube rotator (Fisher Scientific) for 24 hours or until fully dissolved. Prior to bioprinting, the tube was centrifuged at 2000 x g for 5 minutes to remove all bubbles.

Afterwards, the bioink is carefully loaded into the cartridge and placed into the fridge for 15 minutes prior to bioprinting, in sterile conditions. The bioprinting is carried out using a syringe pump printhead (Cellink, Sweden), with an extrusion rate of 3 s^{-1} for the gelatin (all concentrations) and $6\text{ }\mu\text{L s}^{-1}$ for the methylcellulose and print speed of 2 mm s^{-1} .

All steps were performed under aseptic conditions. μT suspensions were recovered from agarose microwell arrays and passed through a $300\text{ }\mu\text{m}$ PluriStrainer (PluriSelect) to remove agarose debris while allowing μT s ($\approx 180\text{ }\mu\text{m}$ diameter) to pass. The filtrate was transferred to a conical tube and centrifuged at 650 g for 5 min to pellet the μT s. The μT tube and a separate tube containing sterile 1% (w/v) gelatin were placed on ice. The supernatant was carefully aspirated to obtain a dry μT pellet, after which the appropriate volume of cold 1% gelatin/ 2% RT methylcellulose was added using a positive-displacement pipette (Microman, Gilson) to minimise bubble formation. The suspension was gently mixed by slow pipetting until homogeneous. The resulting bioink was loaded into sterile printing syringes, placed in a 120 mm Petri dish (ThermoFisher), and kept in a refrigerator for $15\text{--}20\text{ min}$ to allow complete gelation before bioprinting. The printing parameters for μT bioprinting are described in the above paragraph.

4.2.3 Modification of Xanthan Gum with methacrylic group (1H-NMR) and support bath preparation

XG (Sigma) was dissolved at 0.5% (w/v) in ultra-pure water and stirred until completely dissolved. Once dissolved, glycidyl methacrylate (40 mL) was added to the XG solution and stirred overnight at $60\text{ }^{\circ}\text{C}$, protected from light. The solution was now dialysed for 7 days against DI with water changes twice a day, freeze-dried for 48 hours , and stored at $-20\text{ }^{\circ}\text{C}$ for long-term storage. To ensure sterilization, aliquots of XG-MA were weighted and placed into a 50 mL tube and sterilized in an ethylene oxide gas sterilizer (Andersen Products) for 12 hours . 2 days prior printing, the material was dissolved into phenol free DMEM (Gibco) supplemented with penicillin (100 U mL^{-1}), streptomycin ($100\text{ }\mu\text{g mL}^{-1}$) (P/S) (both Gibco) and let it rotate at 40 rpm at RT for 2 days . The day of printing, Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (Sigma), was added for a final concentration of 0.25% (w/v) into the XG-MA solution and vortexed. To ensure the removal of the bubbles, the bath was centrifuged at $2500\times\text{ g}$ for 5 minutes at RT, before printing. For each print, XG-MA (4

mL) was pipetted into a 6-well plate with a pipette for viscous liquids (Microman E, Gilson) to ensure no formation of bubbles during the pipetting.

4.2.4 Rheological assessment of bioinks and xanthan gum-based support bath

Rheological tests were performed on an MCR 102 rheometer (Anton Paar) with Peltier temperature control using a 25 mm parallel-plate geometry (PP25). Flow curves (viscosity vs shear rate, $0.1\text{--}1000\text{ s}^{-1}$) were acquired at $4\text{ }^{\circ}\text{C}$ for gelatin and at room temperature (RT) for methylcellulose and XG-MA support baths. An amplitude sweep at RT identified the linear viscoelastic region (LVR) and yield stress. Frequency sweeps at RT ($\omega = 1\text{--}100\text{ rad s}^{-1}$) were used to obtain storage (G') and loss (G'') moduli. Thixotropy was assessed at RT using a three-interval step test: 0.03 s^{-1} for 30 s, 10 s^{-1} for 30 s, then 0.03 s^{-1} for 30 s, with viscosity recorded throughout. Samples were maintained in a high-humidity environment to prevent dehydration. All measurements were performed in triplicate.

4.2.5 Live/Dead imaging

Constructs were rinsed in $1\times$ PBS and incubated for 1 h at $37\text{ }^{\circ}\text{C}$ with calcein AM ($2\text{ }\mu\text{M}$) and ethidium homodimer-1 (EthD-1, $4\text{ }\mu\text{M}$) (Invitrogen). After three PBS washes, samples were imaged on a Leica SP8 confocal microscope. Calcein was excited at 488 nm and collected at $\sim 530\text{ nm}$; EthD-1 was excited at 530–543 nm and collected at $\sim 645\text{ nm}$. Maximum-intensity z-projections (top-down) were generated to assess viability through the construct depth.

4.2.6 Biochemical analysis

For construct analysis, constructs were retrieved by mechanically disrupting the surrounding support bath and carefully extracting the samples using tweezers. Samples were washed twice in $1\times$ PBS, dried, and digested at $60\text{ }^{\circ}\text{C}$ for 18 h in papain solution (3.88 U/mL papain, 100 mM sodium phosphate, 5 mM Na_2EDTA , 10 mM L-cysteine, pH 6.5; all Sigma). DNA content was quantified using the Quant-iT PicoGreen dsDNA kit (Molecular Probes, Biosciences) according to the manufacturer's instructions. Sulphated glycosaminoglycans (sGAG) were measured by dimethylmethylene blue (DMMB) assay with the dye adjusted to pH 1.5 to minimise background. A chondroitin sulphate standard (1

mg/mL; Sigma) was used, and absorbance at 530 and 590 nm was recorded on a Synergy HT microplate reader (BioTek). The 530/590 ratio was used to generate the standard curve and calculate sGAG content. Total collagen was determined via hydroxyproline (Hyp) quantification using a chloramine-T/DMAAB assay. Briefly, samples were mixed 1:1 with 38% HCl and hydrolysed at 110 °C for 18 h, cooled, centrifuged (5,000 × g, 5 min), dried at 60 °C for 48 h, and reconstituted in 200 µL ultrapure water. Chloramine-T (2.82% w/v) and 4-(dimethylamino)benzaldehyde (0.05% w/v) were added and incubated for 20 min at room temperature in the dark. Absorbance at 570 nm was measured (Synergy HT) against a trans-4-hydroxy-L-proline standard curve, and collagen content was calculated using a Hyp: collagen mass ratio of 1:7.69.

4.2.7 Histological analysis

Samples were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated through graded ethanol (50–100% v/v), cleared in xylene, and paraffin-embedded. Sections (5 µm) were rehydrated and stained with H&E for morphology. Sulphated GAGs were visualised with 1% (w/v) Alcian Blue 8GX in 0.1 M HCl, with 0.1% (w/v) nuclear fast red counterstain; collagen was assessed with 0.1% (w/v) Picrosirius Red (all Sigma). Sections were mounted in DPX and scanned on an Aperio ScanScope (Leica, Germany). Immunohistochemistry for collagen I (Abcam, ab90395; 1:400), collagen II (Santa Cruz, sc-52658; 1:400), and collagen X (Abcam, 49945; 1:200) was performed as previously described [277].

4.2.8 Statistical analysis

Statistical analyses were performed using the software package GraphPad Prism (version 10.4). Statistical tests used to assess the normal distribution of data or to compare groups are indicated in Fig. legends. Group differences were evaluated with one-way ANOVA; group-by-time effects with two-way ANOVA. When ANOVA was significant, Tukey's post hoc multiple comparisons test was applied. Significance was set at $p < 0.05$. All graphs were generated in GraphPad Prism.

4.3 Results

4.3.1 μ Ts were easily extruded when encapsulated into 5% gelatin or 2% methylcellulose

To support the bioprinting of μ Ts, the printability of two different sacrificial bioinks- gelatin and methylcellulose was explored. Both inks are known to be cytocompatible and have been widely used in bioprinting [317, 318]. The rheological properties of the two bioinks were first investigated to check their shear-thinning properties and viscosity with increasing shear rate. The rheological behaviour of the two bioink formulations was characterised by measuring shear stress as a function of shear rate (Fig. 4.1A).

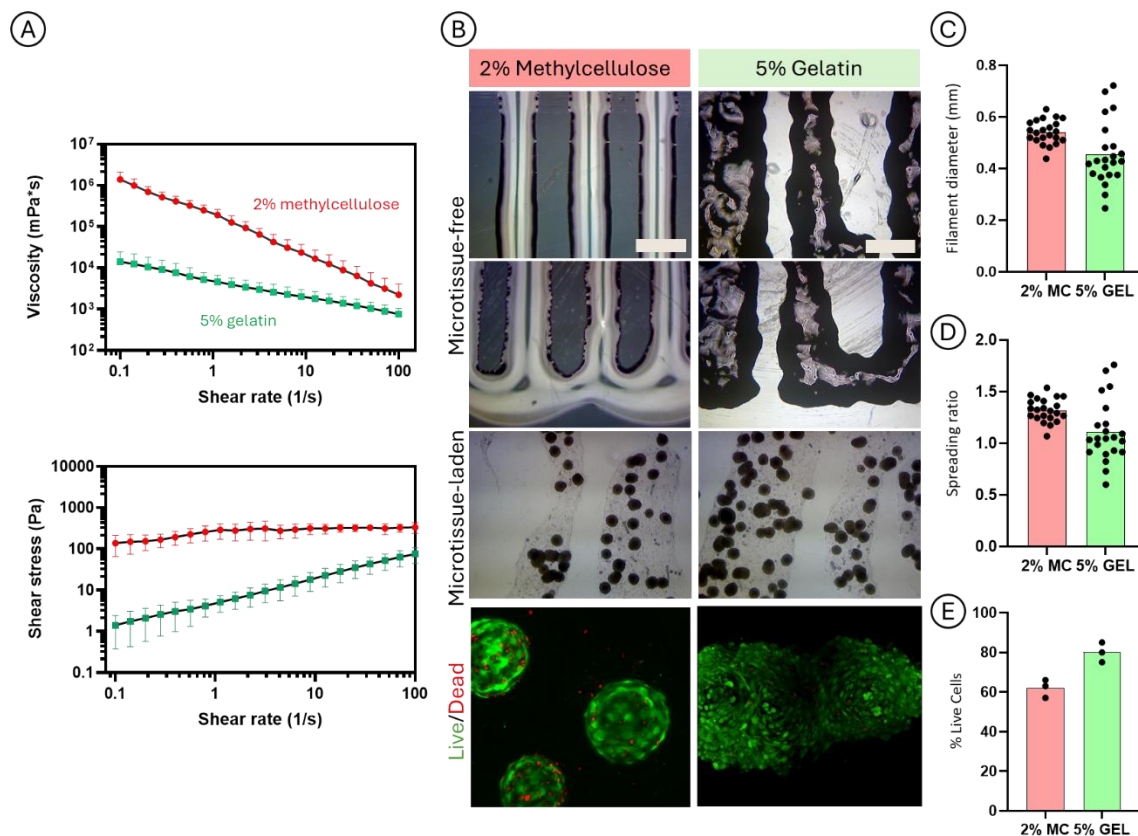


Figure 4.1. Bioinks characterisation. A) Viscosity and shear stress profiles of 2% methylcellulose and 5% gelatin measured over a shear rate range of 0–1000 s^{-1} at 4 $^{\circ}\text{C}$. B) Representative prints of bioinks in a grid pattern (scale bar: 100 μm). μT -free: bioinks printed without cellular components. μT -laden: bioinks containing μT s at a density of 10,000 μT s/mL. μT viability was evaluated 7 days post-printing. C) Filament diameter and spreading ratio (D) of printed filaments containing μT s. E) Percentage of live cells relative to total cells, quantified using ImageJ. Live cell percentage was normalised to the total cell percentage.

Both formulations exhibited shear-thinning behaviour, evidenced by a decrease in viscosity with increasing shear rate, which is typical and desirable for extrusion-based bioprinting applications. As methylcellulose had four times higher viscosity (10^8 mPa *s) compared to 5% gelatin (10^4 mPa* s), a higher extrusion pressure was needed to extrude the bioink, which would cause the μ Ts to experience higher shear stress (Fig. 4.1B). Therefore, while both bioinks are shear-thinning and suitable for extrusion, 2% methylcellulose may be better suited for applications requiring greater structural integrity, while 5% gelatin may be advantageous for cell viability due to lower extrusion forces.

Following rheological analysis, both bioinks were subjected to extrusion-based bioprinting. 2% methylcellulose required a higher extrusion (20 kPa) pressure due to its higher viscosity; however, the resulting filaments exhibited smooth edges (Fig. 4.1B), indicating that the applied shear stress did not induce visible structural deformation during printing (Fig. 4.1B). In contrast, 5% gelatin required a lower extrusion pressure compared to 2% methylcellulose, but the printed filaments displayed irregular, ridged edges with curled features caused by greater shear-induced deformation during extrusion (Fig. 4.1B). Despite these differences, both bioinks demonstrated high print fidelity, maintaining well-defined geometries post-printing. To further evaluate their suitability for more complex designs, both bioinks were used to print μ Ts on a flat surface in a grid pattern (Fig. 4.1B). Each bioink successfully extruded μ Ts at a density of approximately 10,000 μ Ts per mL of bioink. Post-printing, the μ T distribution in the filament appeared to be homogeneous along the length of the printed filament (Fig. 4.1B). The μ Ts, with an average diameter of ~ 180 μ m, were printed through a 22G nozzle (410 μ m inner diameter) without clogging (Fig. 4.1B). Filament diameter was assessed for both μ T-laden bioinks, showing a filament diameter close to 500 μ m and a spreading ratio slightly higher than 1 (Fig. 4.1C, D). Both bioinks were compatible with μ T printing, and successful extrusion required matching μ T size to the nozzle diameter to prevent clogging. Cell viability within the printed μ Ts was assessed 7 days post-printing (Fig. 4.1B). Both bioinks supported high levels of viability, with no significant differences observed between 2% methylcellulose ($\sim 60\%$) and gelatin ($\sim 80\%$) ($p > 0.05$) (Fig. 4.1E).

4.3.2 μ T-laden gelatin bioinks maintain their chondrogenic potential following bioprinting into a FRESH support bath

After evaluating the rheological properties of the two bioinks and confirming μ T viability post-printing, we next assessed the chondrogenic potential of μ Ts printed with each material using a conventional FRESH support bath (Fig. 4 .2). The FRESH bath is composed of a thermoreversible gelatin microparticle slurry, which acts as a sacrificial support during printing [202]. Upon warming to 37 °C, the gelatin slurry liquefies and can be removed, leaving the printed construct intact for subsequent cell culture.

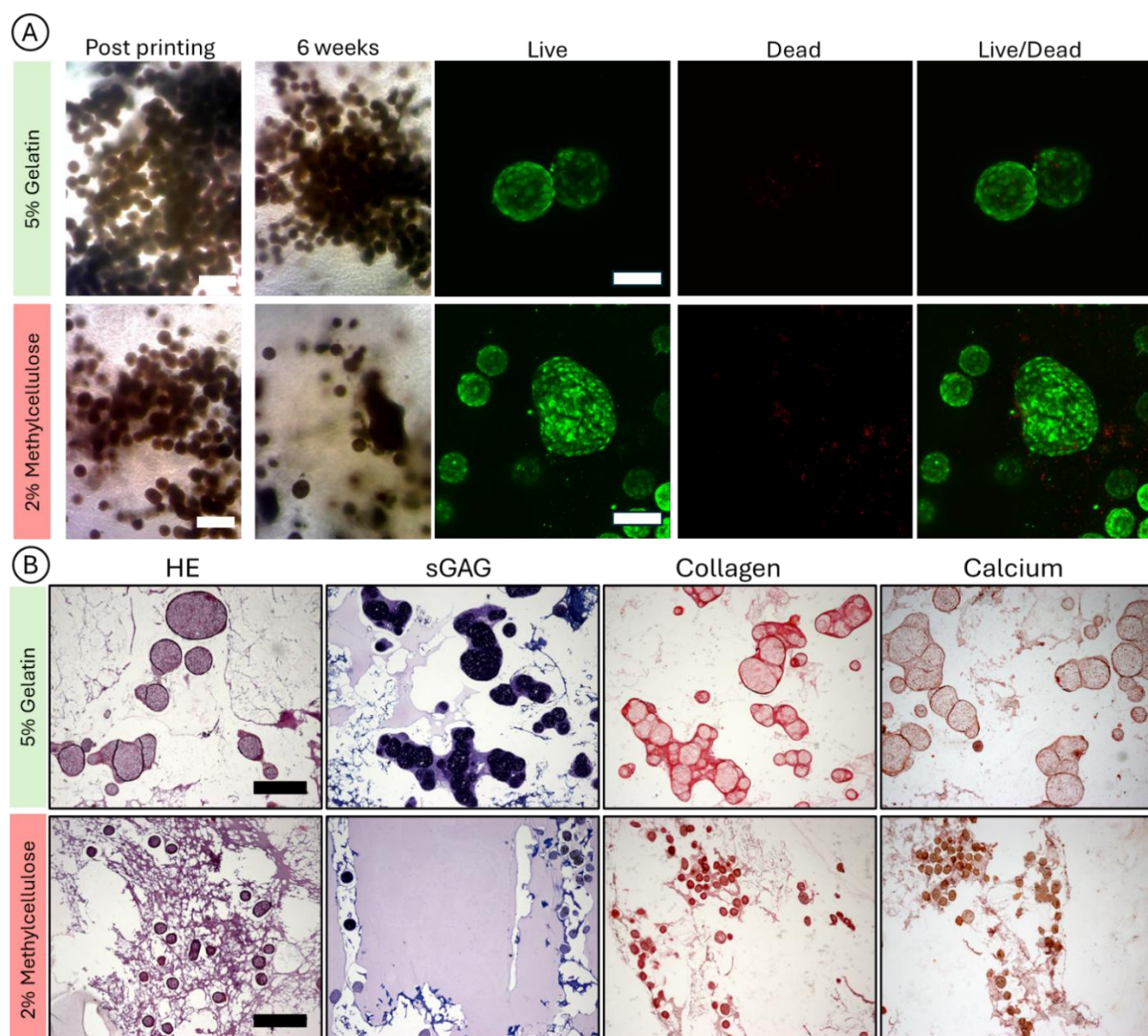


Figure 4.2. Gelatin bioink supports chondrogenesis better than methylcellulose. A) Brightfield microscope pictures of bioprinted μ Ts into a FRESH support bath with gelatin (green) and methylcellulose (red), and Live/Dead showing high viability of printed μ Ts 7 days post printing with both bioinks. Scale bar (brightfield microscope): 500 μ m. Scale bar (Live/Dead): 150 μ m. B) Histological staining for bioprinted μ Ts at 4 weeks

post-printing in gelatin and methylcellulose. μ Ts encapsulated into a gelatin bioink show more intense staining for GAGs and better μ T fusion. Scale bar: 610 μ m.

Both bioinks enabled extrusion of the μ Ts through the needle and were adequately supported by the FRESH bath during printing (Fig. 4.2A). However, after four weeks of chondrogenic culture, μ T fusion remained incomplete in both conditions, despite high cell viability observed for both bioinks at day 7 post-printing (Fig. 4.2A). Histological analysis was performed using classical cartilage stains, including Alcian blue for sGAGs and Picrosirius Red for collagen visualisation (Fig. 4.2A). μ Ts printed with methylcellulose remained smaller in diameter (Fig. 4.2B) and exhibited limited fusion, whereas those printed with gelatin demonstrated more extensive fusion, with larger and more variable μ T sizes (Fig. 4.2B). Furthermore, sGAG staining was markedly more intense in the gelatin group, suggesting enhanced chondrogenic potential (Fig. 4.2B). In contrast, the methylcellulose group showed weak Alcian blue staining and, notably, positive staining for calcium deposition, indicating a potential shift toward osteogenic rather than chondrogenic differentiation (Fig. 4.2B). The high viscosity and poor degradability of methylcellulose within the FRESH support bath likely impeded close contact between μ Ts, hindering the necessary μ T interactions required for fusion. Conversely, gelatin—being more readily washed out of the support bath—facilitated faster contact between μ Ts, promoting fusion and cartilage matrix deposition. Given the critical importance of μ T fusion in engineering large-scale cartilaginous grafts, and the absence of unwanted calcium deposition in the gelatin condition, we selected gelatin as the preferred bioink for continued development of this μ T bioprinting platform.

4.3.3 μ Ts were successfully bioprinted into 1% gelatin bioink at different μ T densities into a support bath

To improve μ T printing and ensure better flow of the bioink while extruding, and due to challenges encountered when printing μ Ts suspended in 5% gelatin at higher μ T densities, we investigated the printability of acellular gelatin solutions at varying lower concentrations. We hypothesised that the high viscosity of the 5% gelatin formulation may have impaired proper extrusion, thereby compromising the fidelity of the printed

constructs. To evaluate this, we tested gelatin concentrations ranging from 5 to 1 wt%, examining their rheological behaviour and printability (Fig. 4.3A).

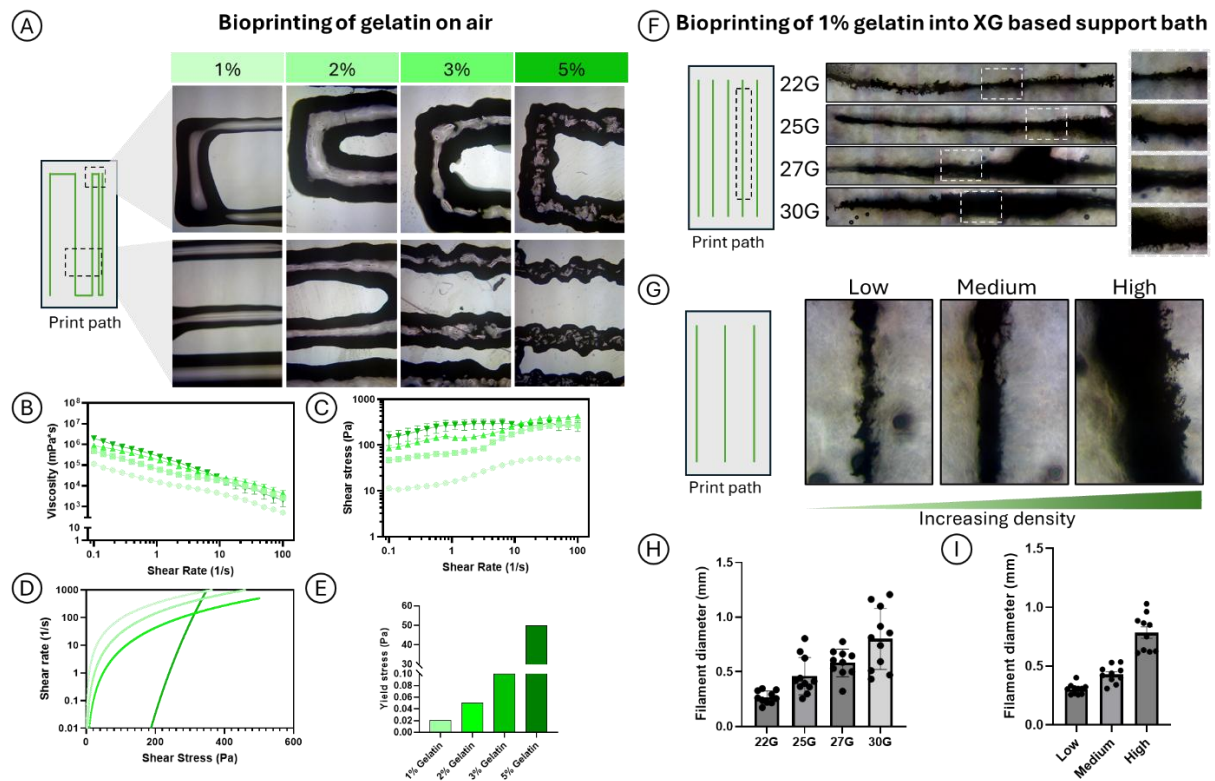


Figure 4.3. Printability of gelatin at different concentrations. A) Brightfield microscope pictures of gelatin bioink bioprinted in a grid pattern on a flat surface. Scale bar: 200 μ m. B) Viscosity and C) shear stress profiles of gelatin bioinks measured over a shear rate range of 0–1000 s^{-1} at 4 $^{\circ}C$. D) Stress ramp curves for gelatin bioinks at different concentrations. E) Yield stress of gelatin bioinks. F) Brightfield microscope pictures of 1% gelatin printed into the xanthan gum support bath at different needle gauges. G) brightfield microscope pictures of 1% gelatin mixed with a particle-based ink at various densities (low, medium, high). H) Filament diameter measurements of 1% gelatin printed into XG support bath with different needle gauge (referred to panel F), and I) filament diameter of 1% gelatin mixed with particle-based ink at various densities (referred to panel G).

Across this range, all formulations exhibited favourable rheology, including shear-thinning behaviour and low shear stress requirements during extrusion (Fig. 4.3). Specifically, 1% and 2% gelatin solutions exhibited minimal resistance to flow with very low yield stress (Fig. 4.3B), consistent with their low viscosity and high fluidity. The 3% gelatin formulation displayed moderate structural integrity with a steeper viscosity-shear rate profile, suggesting increased resistance to flow (Fig. 4.3B). In contrast, the 5% gelatin formulation

exhibited pronounced viscoplastic behaviour, characterised by a delayed onset of flow and a sharp increase in shear rate beyond a critical stress threshold. This behaviour reflects the formation of a strong internal network structure, likely accounting for the rigid, ridged patterns observed during printing with this formulation.

Based on these findings, 1% gelatin was selected as the optimal compromise between ease of printability and thermal sensitivity, as lower concentrations liquefy at lower temperatures, offering practical advantages for extrusion. The extrusion of 5% gelatin required higher pressures to reach the yield stress (Fig. 4.3B), resulting in filament deformation and irregular surface topography (Fig. 4.3A).

The use of the FRESH support bath enabled μ T bioprinting; however, its sacrificial nature did not allow retention of the printed constructs with high geometric fidelity. Furthermore, when comparing a commercially available FRESH bath with an in-house synthesised version, we observed significant variability between batches, likely due to differences in synthesis conditions. This lack of reproducibility limited its suitability as a reliable support material. To overcome these issues, we investigated xanthan gum as an alternative support bath, given its low cost, ease of synthesis, and potential to provide more consistent performance [319]. Subsequently, we proceeded to print 1% gelatin using a syringe pump and a xanthan gum-based support bath, testing needles of various gauges. All prints were carried out under consistent parameters (extrusion speed: 6 mm/s; travel speed: 3 mm/s). Interestingly, increasing the needle gauge (i.e., decreasing the internal diameter) resulted in filaments with larger diameters and greater spreading ratios (Fig. 4.3F, H).

To simulate the effect of increased μ T density on filament formation, we performed a bioprinting experiment using a gelatin-based ink containing black microparticles. The particle-laden ink was formulated by increasing the volume fraction of black particles while maintaining the gelatin concentration at 1%. As the particle density increased, we observed a corresponding increase in filament diameter under identical printing conditions (Fig. 4.3G, I). This suggests that higher μ T densities may similarly influence filament geometry, potentially reducing printing resolution in complex constructs if not properly controlled.

We then evaluated μ T-laden inks at different cell densities (ranging from 2,000 to 60,000 μ Ts/mL, corresponding to approximately 4×10^6 to 120×10^6 cells/mL) printed into a

xanthan gum-based support bath. The primary aim was to assess μ T fusion behaviour at varying densities (Fig. 4.4).

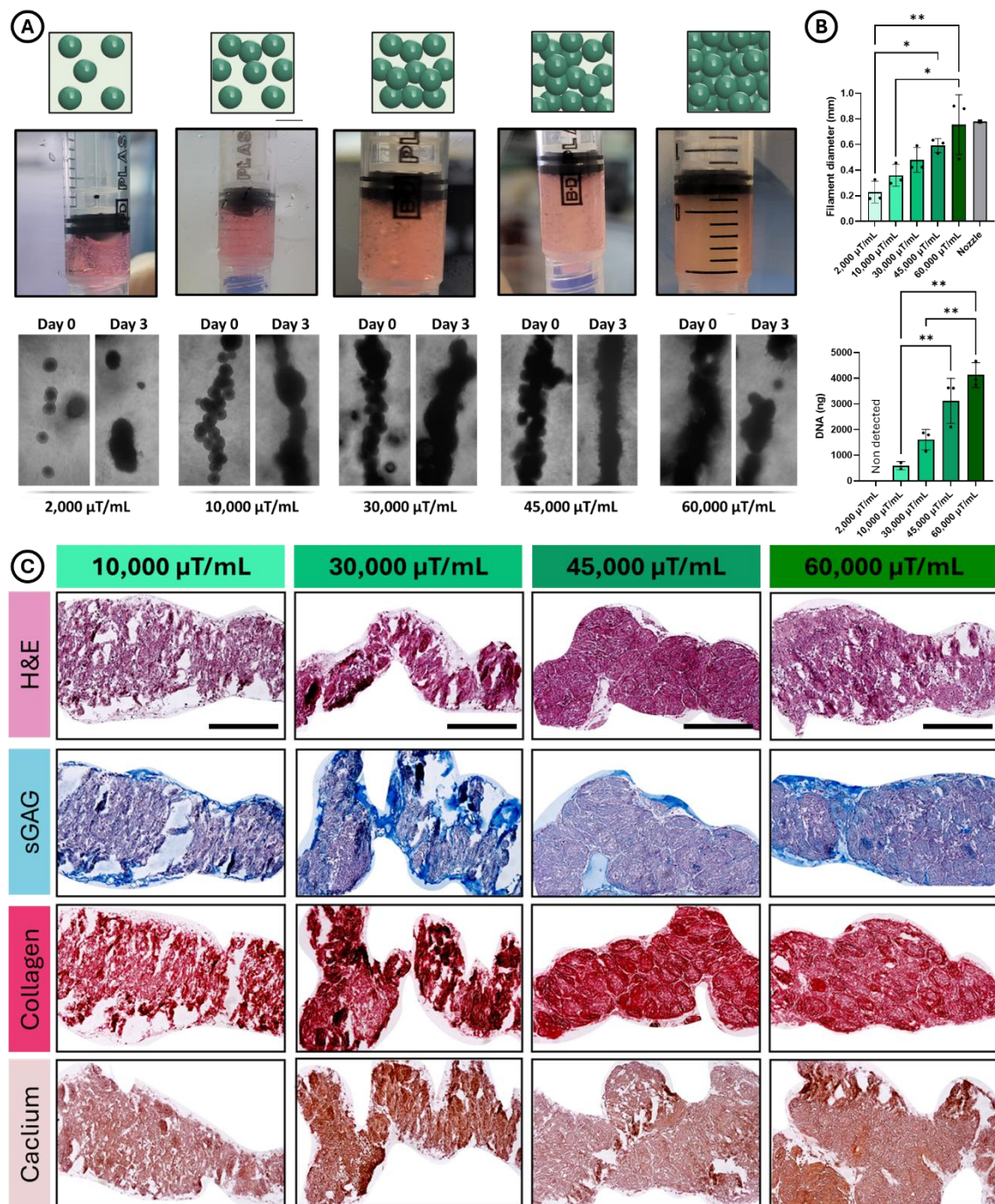


Figure 4.4. μ Ts were extruded into a support bath at varying densities, showing improved fusion at higher densities. A) Macroscopic images of μ T-laden bioinks at increasing densities within the printing cartridge, with corresponding brightfield images of printed μ Ts arranged in a filament pattern at day 0 and day 3, highlighting fusion over time. B) Filament diameter measurements at day 0 and DNA quantification by biochemical assays compared to nozzle diameter (grey). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$). C)

Histological staining for haematoxylin & eosin (H&E), sGAG (AB), collagen (PR), and calcium deposits (AR). Scale bar: 400 μm .

At lower densities, μT s were sparsely distributed, leading to inconsistent tissue filament formation (Fig. 4.4A). In some regions, fusion occurred (Fig. 4.4A), while in others, μT s remained isolated within the support bath, preventing effective fusion (Fig. 4.4A, 2,000 μT s/mL). As the μT density increased, filament consistency improved. However, heterogeneity in μT distribution across the cartridge likely contributed to non-uniform filament compositions in intermediate densities (Fig. 4.4A). The most consistent and uniform filaments were obtained at a concentration of 45,000 μT s/mL (equivalent to 90×10^6 cells/mL) (Fig. 4.4A). At this density, μT s were evenly distributed along the filament and demonstrated successful fusion after three days of incubation (Fig. 4.4A). Higher densities (60,000 μT s/mL) led to clogging issues during extrusion, resulting in disrupted filament continuity and structural gaps (Fig. 4.4A). The improved filament consistency in all groups was further supported by an increase in DNA content, as determined by quantitative DNA assays. Finally, the 45,000 μT s/mL density was selected as the optimal formulation for all subsequent printing experiments presented in this thesis, offering the best balance between print fidelity, μT fusion, and reliable extrusion performance (Fig. 4.4).

4.3.4 A comparison of XG and XG-MA as a support bath for the bioprinting of μT s

To support the long-term culture of bioprinted μT s, we initially employed a XG-based support bath. μT -laden filaments were printed into the XG bath and subsequently cultured in chondrogenic media for a minimum of four weeks. However, the XG bath exhibited limited temporal stability, gradually dissolving and washing away within 7–10 days of culture. This temporary, sacrificial behaviour of the material resulted in a progressive loss of mechanical support for the printed constructs during the critical post-printing period. As the bath degraded, the printed structures were no longer mechanically stabilised, coinciding with the onset of chondrogenic differentiation. During this process, the embedded cells interacted robustly via cell–cell and cell–ECM signalling to form a cohesive tissue. These dynamic interactions, in the absence of sustained mechanical confinement,

led to pronounced tissue shrinkage over time, resulting in a significant reduction in construct dimensions relative to the initial printed geometry. This shrinkage was associated with a tissue phenotype undergoing fibrochondrogenesis and hypertrophy, as indicated by positive staining for collagen type I and X across all densities and absent staining for collagen type II (Fig. S 4.1).

Therefore, we sought to develop a more persistent support material that would maintain structural integrity throughout the entire culture period. Accordingly, we functionalized xanthan gum with methacrylate groups to enable photo-crosslinking post-printing (Fig. 4.5).

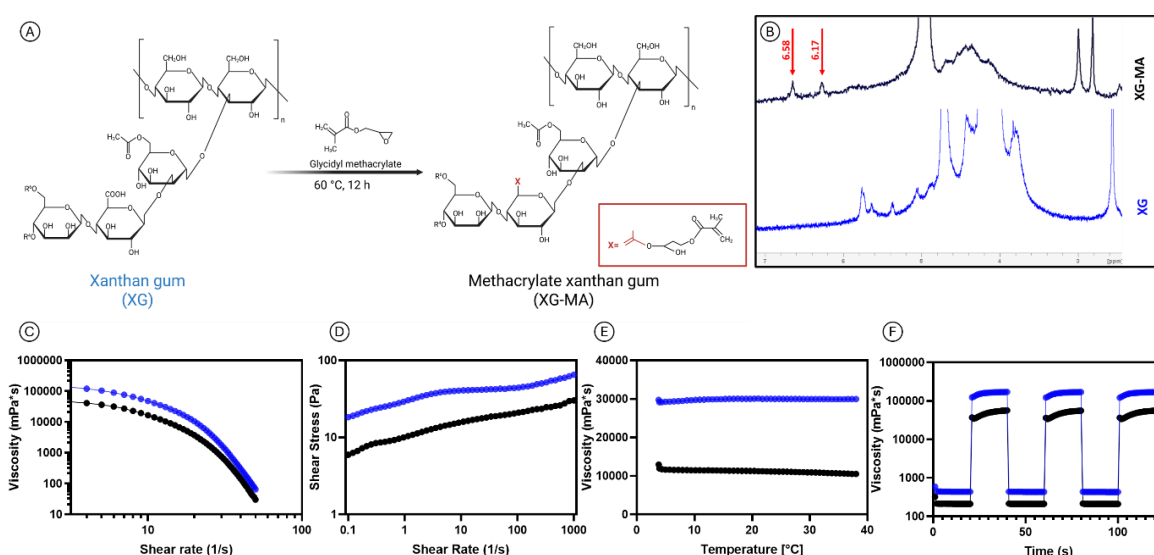


Figure 4.5. Xanthan gum (XG) and methacrylate xanthan gum (XG-MA) were successfully synthesised, and they show good rheological properties suitable for support baths. A Schematic of XG modification with glycidyl methacrylate (created with BioRender). B) ^1H -NMR spectra of xanthan and methacrylate XG. The peaks highlighted with the red arrows correspond to the peaks (6.58 and 6.17 ppm) of the methacrylate group attached to the XG backbone. C) Viscosity and D) shear stress profiles XG-based baths measured over a shear rate range of 0–1000 s⁻¹ at room temperature. E) Viscosity profiles measured at temperatures between 4–38 °C, showing stable viscosity across conditions, confirming suitability for printing at physiological temperatures to support cell viability. F) Viscosity profiles under low (0 s⁻¹) and high (1000 s⁻¹) shear rates across multiple cycles, demonstrating the thixotropic behaviour of the baths.

The methacrylation of xanthan gum was achieved using glycidyl methacrylate, following the protocol described by Patricio *et al.* (2020) [232] (Fig. 4.5 A). The success of the chemical

modification was confirmed via NMR analysis (Fig. 4.5 B), where two characteristic peaks at 6.58 ppm and 6.17 ppm (red arrows, Fig. 4.5 B) indicated successful incorporation of methacrylate moieties, in contrast to the unmodified XG spectrum. Rheological characterisation of both XG and XG-MA support baths at 1% (w/v) demonstrated that methacrylation did not compromise the shear-thinning properties essential for μ T extrusion (Fig. 4.5 C-G). However, a slight increase in viscosity was observed in the XG-MA formulation, which remained within a printable range (Fig. 4.5 C). To assess the impact of support bath chemistry on tissue maturation, we printed μ T-laden bioinks at the same cell density into both XG and XG-MA baths and cultured the constructs for 21 days in chondrogenic media (Fig. 4.6). Before bioprinting, we first assessed the microtissue viability post-encapsulation with 1% gelatin, showing high viability of microtissues before printing (Fig. S 4.2).

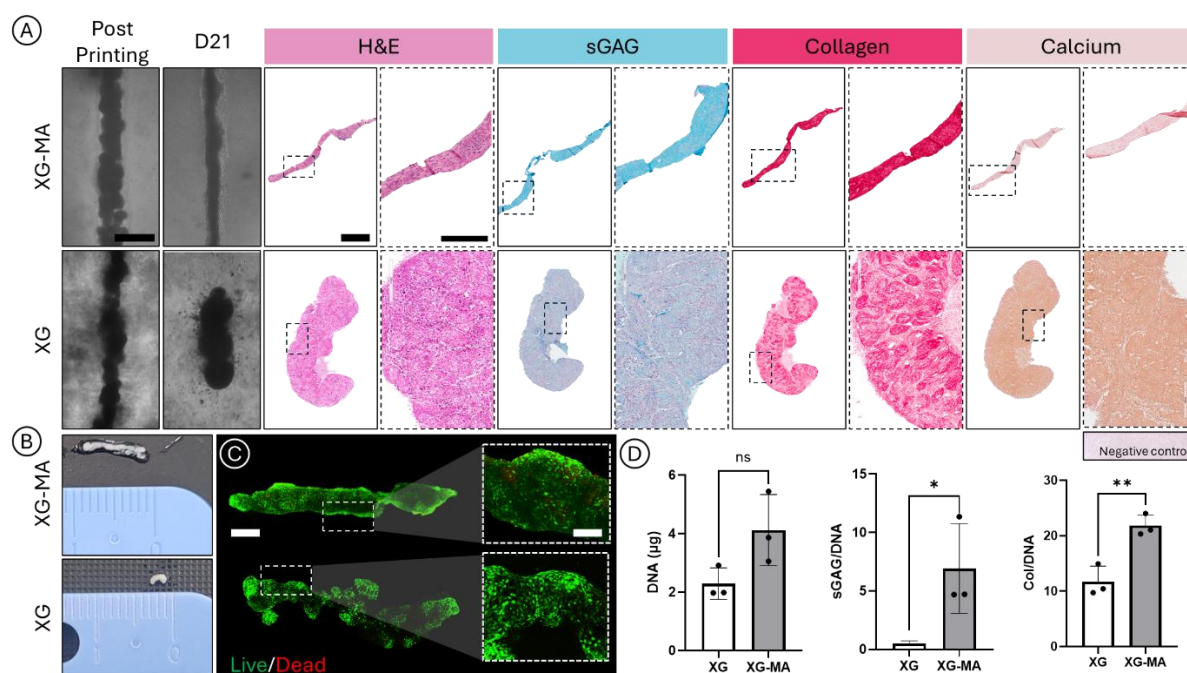


Figure 4.6. XG-MA support bath limits the contraction of μ T-laden bioprinted constructs and exhibits enhanced chondrogenesis compared to XG. A) Brightfield images of μ Ts printed in XG and XG-MA support baths immediately after printing and after 21 days of culture (45,000 μ Ts/mL) (Scale bar: 200 μ m), alongside histological staining for H&E, sulphated glycosaminoglycans (sGAG), collagen, and calcium deposition. Scale bars: 500 and 100 μ m. B) Macroscopic images of printed μ T filaments after 21 days in chondrogenic medium. C) Live/dead staining of printed μ Ts stained with Ethidium bromide (Dead) and calcein (Live). Scale bars (from

left to right): 300 μ m and 50 μ m. D) Biochemical quantification of DNA, sGAG content and collagen content related to DNA. Statistical analysis was performed using an unpaired *t*-test, with significance accepted at $p < 0.05$. (* $p < 0.05$, ** $p < 0.01$).

Both groups exhibited high cell viability at 7 days post-printing, suggesting that the chemical modification did not adversely affect cell viability (Fig. 4.6C). However, notable differences were observed in the macroscopic appearance, histological staining, and biochemical composition of the resulting tissues. Constructs printed in the unmodified XG bath underwent significant contraction over the culture period, with clear reductions in filament length (Fig. 4.6 A). In contrast, constructs printed in the XG-MA bath maintained their original dimensions throughout the entire 21-day culture period, preventing tissue shrinkage (Fig. 4.6 A). Constructs cultured in XG-MA demonstrated more intense and homogeneously distributed staining for glycosaminoglycans (Alcian Blue) and collagen (Sirius red), indicative of enhanced chondrogenic differentiation (Fig. 4.6 A). The persistent support provided by XG-MA not only preserves construct geometry but also favours chondrogenic over osteogenic differentiation. Biochemical quantification confirmed that glycosaminoglycan content was significantly higher in the XG-MA group compared to the unmodified XG group ($p < 0.05$) (Fig. 4.6D).

Furthermore, polarised light microscopy revealed a more organised collagen fibre network in XG-MA constructs, with fibres oriented along a predominant direction (Fig. 4.7 A, B). In contrast, collagen fibres in the XG group appeared randomly oriented and less dense under equivalent imaging conditions (Fig. 4.7 A, B). Coherency analysis of polarised light images showed that collagen fibres in μ Ts bioprinted within XG-MA support baths were predominantly aligned in a single direction, indicating anisotropy, whereas tissues printed in unmodified xanthan gum baths exhibited a more random collagen organisation (Fig. 4.7 B, C). Specific collagen deposition differed between the 2 groups, where μ Ts printed in the XG-MA bath showed homogeneous and positive staining for collagen type I, II (Fig. 4.7 D), while μ Ts printed in the XG bath showed positive staining for collagen type I and X and negative staining for collagen type II (Fig. 4.7 D).

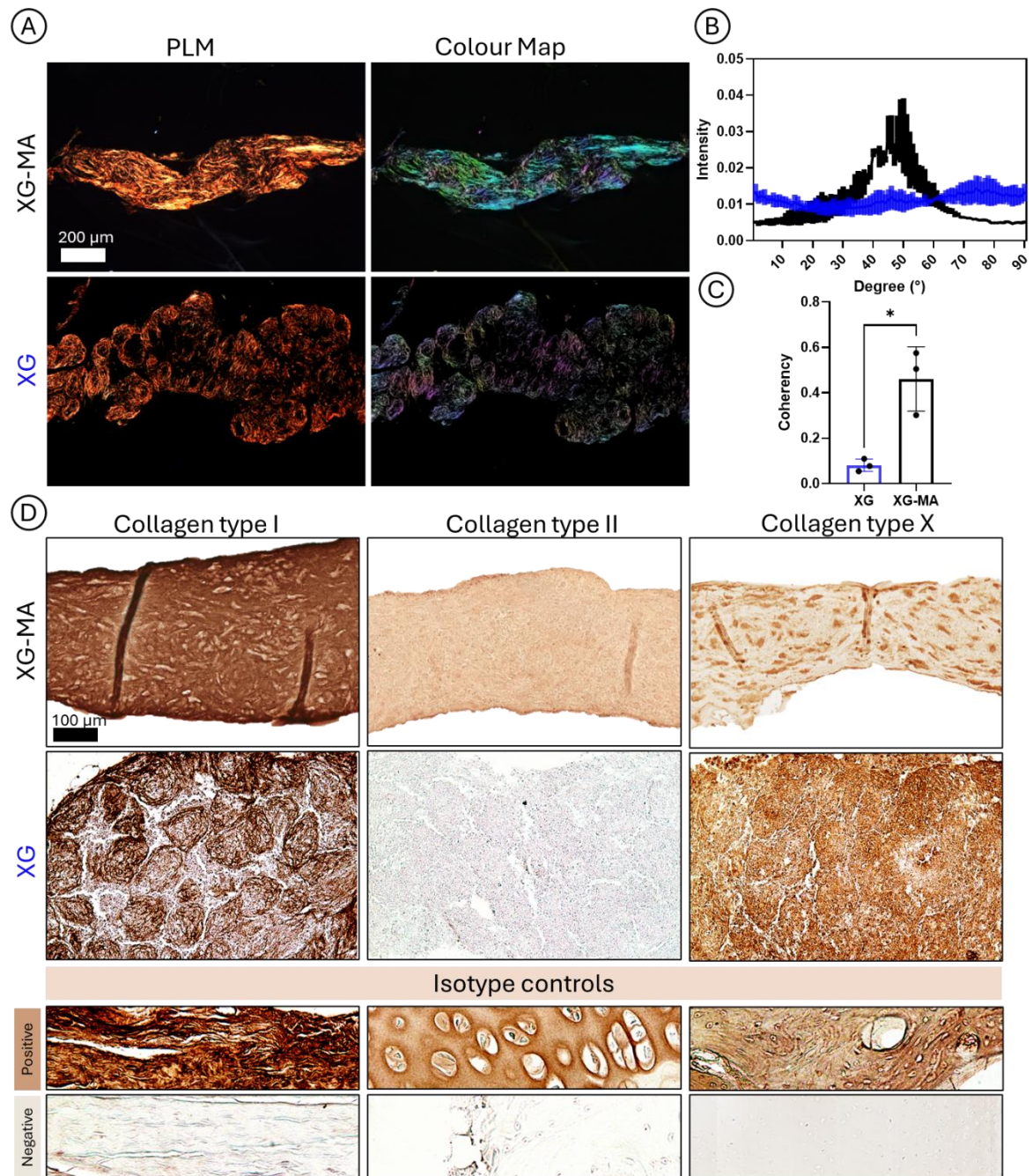


Figure 4.7. The collagen fibre organisation of printed μT s can be better guided using a XG-MA support bath.

A) PLM and colour map for the respective PLM image show a comparison of the spatial organisation of the collagen network formed by the μT s within the support bath over 4 weeks in culture in chondrogenic media. Sb: 200 μm . B) quantification of the directionality of the collagen fibres within the printed μT s and c) fibre coherency, where a value of 1 indicates that fibres are aligned in the same directions, and 0 indicates dispersion of the fibres in all directions. Significant differences were tested using an unpaired t-test. Significance was accepted when $p < 0.05$. D) DAB immunohistochemistry showing collagen types I, II, and X within the printed filaments. Isotype controls included ligament sections with and without primary antibody for collagen type I; articular cartilage (AC) with and without primary antibody for collagen type II; bone as a

positive control for collagen type X; and articular cartilage as a negative control for collagen type X. Sb: 100 μm .

4.4 Discussion

The primary advantage of using μT s in TE lies in their ability to synthesise their own ECM, secrete tissue-specific soluble factors, and be generated at a large scale using automated platforms [33, 161, 172, 175, 307]. Furthermore, μT s possess an inherent capacity to fuse, making them ideal modular building blocks for constructing more complex tissue structures through emerging biofabrication and bioprinting approaches [36]. Despite these advantages, there remains a significant gap in the literature regarding the large-scale bioprinting of μT s at high densities. Currently, no standardised method or technique has been established to reliably print μT s at high throughput while preserving viability and promoting fusion and driving tissue maturation. Leveraging our expertise in high-throughput μT fabrication, we sought to address this gap by developing a novel method for bioprinting μT s at high densities. This study investigated the critical parameters influencing successful extrusion-based bioprinting and subsequent maturation of μT -laden constructs for cartilage TE. Specifically, we assessed the printability, rheological behaviour, and biological outcomes of two candidate bioinks (gelatin and methylcellulose), in combination with various support baths (FRESH, xanthan gum, and XG-MA), and explored how variables such as gelatin concentration and μT density impacted overall performance.

Our findings highlight the complex interplay between bioink rheology, support bath cytocompatibility, and cellular behaviour—particularly as these factors influence μT fusion and the transition from single μT units to cohesive, functional engineered cartilaginous tissues. Previous studies have demonstrated the feasibility of μT printing; however, only recently did Kronemberger *et al.* (2024) illustrate the critical role of rapid hydrogel degradation in facilitating post-printing μT fusion [66]. Specifically, they showed that oxidised alginate, which degrades within 7–10 days [218], enabled the printed μT s to come into closer contact and initiate fusion, leading to the formation of larger, more continuous tissue constructs. Based on this insight, it was hypothesised that employing rapidly degrading or sacrificial hydrogels could similarly promote μT fusion post-printing. While we initially tested OA, its low viscosity resulted in μT sedimentation within the cartridge and frequent nozzle clogging, rendering it unsuitable for this thesis. As an alternative, we

evaluated two more viscous sacrificial materials—2% methylcellulose and 5% gelatin—both of which have been previously explored for extrusion-based bioprinting [320-322]. Rheological analysis confirmed that both materials exhibited shear-thinning behaviour, a key characteristic for extrusion printing. However, notable differences were observed in their absolute viscosities. Methylcellulose displayed viscosities approximately four orders of magnitude higher than gelatin under low shear conditions. While this translated into superior filament shape fidelity and smoother extrusion profiles, it also necessitated significantly higher extrusion pressures. Consequently, μ Ts embedded in methylcellulose were exposed to greater shear stress during printing, resulting in lower post-printing viability (~60%) compared to gelatin (~80%). In contrast, gelatin exhibited more favourable handling characteristics for μ T bioprinting: it required lower extrusion pressures, better preserved μ T viability, and was more readily washed out of the support bath post-printing [211]. However, due to its lower viscosity and yield stress, gelatin resulted in less uniform filaments, with some deformation observed. These findings underscore the critical need to balance mechanical stability with biological compatibility when selecting a bioink for μ T printing. Ultimately, our results suggest that gelatin offers a more viable compromise, supporting both printability and post-printing tissue maturation, particularly in applications targeting cartilage regeneration. When both bioinks were used to print μ Ts into FRESH support baths, differences in long-term biological outcomes became more pronounced. While both inks maintained high viability at day 7 post-printing, histological and biochemical analyses after 4 weeks revealed more extensive fusion and stronger chondrogenic matrix deposition in the gelatin group. Methylcellulose-based inks supported limited μ T fusion, weak glycosaminoglycan staining, suggesting chondrogenesis impairment. These results indicate that the degradability and compatibility of the bioink play a key role in supporting tissue-specific ECM deposition [218, 228, 323] and μ T fusion [66]. Methylcellulose, being more persistent and less easily washed from the support bath, likely inhibited sufficient contact between neighbouring μ Ts. Gelatin, on the other hand, dissolved more readily, facilitating μ T fusion and the emergence of a more homogeneous and compact cartilage-like matrix.

As the relatively high viscosity of the 5% gelatin inks negatively impacted the printing process, this thesis next examined acellular gelatin formulations ranging from 1% to 5%. All

concentrations exhibited shear-thinning behaviour; however, lower-concentration gels (1–2%) showed better extrusion profiles due to reduced yield stress and lower required pressure. At the same time, very low gelatin concentrations (e.g., <1%) pose risks of thermal instability and liquefaction at physiological temperatures. Therefore, 1% gelatin was selected as the optimal formulation, balancing printability, structural retention, and temperature sensitivity.

Interestingly, reducing the nozzle diameter (i.e., increasing needle gauge) did not improve printing resolution, but instead resulted in broader filaments. While this may appear counterintuitive, it can be explained by the displacement-controlled nature of the mechanical printhead used in this study, where extrusion is governed by volumetric flow rather than applied pressure. As nozzle diameter decreases, resistance to flow increases, leading to elevated shear stresses within the bioink[324]. In dense, particle-laden systems such as μ T suspensions, this can promote local aggregation or jamming and disrupt flow uniformity, resulting in filament broadening upon deposition. A similar effect was observed with increasing particle concentration, where higher internal resistance further altered filament geometry. Together, these findings highlight that, in displacement-controlled systems, decreasing nozzle diameter does not necessarily translate to improved resolution when printing complex, particulate bioinks such as μ Ts. Consequently, it was essential to select a needle gauge that both allowed the passage of μ Ts without subjecting them to excessive shear stress [212] and simultaneously balanced ink density to prevent the need for elevated extrusion pressure. Based on these findings, the 22G needle was selected as optimal, as it produced the thinnest reproducible filament. The resulting filament dimensions, on the order of several hundred micrometres, define the practical resolution limit of the system, which is constrained by the size of the μ Ts but remains suitable for guiding tissue-level organisation in musculoskeletal applications. To the best of current knowledge, this is the first report in which μ Ts have been extruded through such a small nozzle diameter. Furthermore, its internal diameter of 410 μ m provided sufficient space for multiple μ Ts (average diameter $\sim$ 180 μ m) to pass simultaneously, minimising the risk of clogging while preserving structural fidelity. Bioprinting experiments using varying μ T densities (2,000–60,000 μ Ts/mL) revealed that intermediate densities ($\sim$ 45,000 μ Ts/mL) achieved the best filament homogeneity and post-printing fusion. Densities below this

range led to spatially sparse distributions and poor fusion, while higher densities introduced clogging issues and disrupted filament continuity. These findings align with prior studies emphasising the importance of optimising cell density for fusion-based tissue formation [325, 326].

To maintain construct geometry during long-term culture, we compared the performance of unmodified XG with a photocrosslinkable derivative, XG-MA, as a support baths. While both materials initially supported μ T-laden filament deposition, their behaviour during extended culture and their rheological profiles were notably different. Rheological characterisation of XG and XG-MA at 1% (w/v) confirmed that both materials retained shear-thinning properties—a key requirement for extrusion-based bioprinting. The viscosity of both baths decreased with increasing shear rate, facilitating smooth needle movement during printing and rapid self-recovery post-extrusion to support the deposited filaments. Importantly, methacrylation did not alter the fundamental shear-thinning behaviour, which was comparable to native XG. However, a slight decrease in viscosity was observed in the XG-MA formulation across the tested shear rate range. This reduction in viscosity is likely attributable to structural and chemical changes introduced during methacrylation. Specifically, the substitution of hydroxyl groups with methacrylate moieties reduces the number of available sites for intermolecular hydrogen bonding, a key contributor to the high viscosity of native XG solutions. These combined effects diminish the cohesive interactions between polymer chains, resulting in a lower-viscosity solution prior to UV crosslinking [327]. Similar trends have been reported in other methacrylate polysaccharides, where partial substitution alters polymer conformation and decreases solution viscosity while retaining printability and structural fidelity post-crosslinking [328-330]. Despite this increase in viscosity, XG-MA remained within a printable range and did not require significant adjustments to printing parameters. The modest viscosity enhancement may even provide improved mechanical stabilisation during the printing process by offering greater yield stress and resistance to deformation, particularly in the post-crosslinking phase. From a structural perspective, XG-MA significantly outperformed XG during the 21-day culture period. While XG is inherently sacrificial and begins to dissolve within the first 7–10 days, XG-MA, upon UV crosslinking, remained stable and maintained the original printed geometry throughout the entire culture duration. This was critical for

preventing construct shrinkage during chondrogenic differentiation, a period during which cells form robust cell–cell and cell–matrix connections that otherwise lead to excessive tissue compaction in the absence of physical restraint. In agreement with this, constructs printed into XG showed visible shrinkage and tissue compaction, with weaker matrix deposition and evidence of undesired calcium deposition. In contrast, constructs embedded in XG-MA demonstrated improved tissue retention, higher sGAG and collagen content, and reduced mineralisation. These differences are attributed not only to the prolonged structural support but also to the slight rheological advantage of XG-MA in resisting filament deformation during printing and maintaining spatial positioning throughout the culture period. Moreover, polarised light microscopy revealed more aligned collagen fibres in XG-MA constructs, further underscoring the ability of the crosslinked support bath to guide tissue organisation. The improved ECM deposition and organisation are likely a result of better mechanical confinement, precise patterning of μ Ts, and enhanced fusion dynamics, all of which are influenced by the support bath's physical properties and post-printing tissue stabilisation.

These results demonstrate that the physical microenvironment—imposed by the support bath—can strongly influence cell fate. By preventing premature deformation and/or excessive tissue compaction, XG-MA allowed μ Ts to maintain their spatial configuration post-printing, promoting more physiological ECM production, collagen network alignment and chondrogenic differentiation.

4.5 Conclusion

Collectively, this study underscores the multifaceted requirements for successful bioprinting of μ T-based constructs. Gelatin emerged as the most favourable bioink due to its balance of printability, cytocompatibility, and quick degradability. Optimising gelatin concentration and μ T density further improved print fidelity and fusion behaviour. Importantly, the use of a crosslinkable support bath (XG-MA) significantly enhanced the structural and functional maturation of printed tissues. Future work should explore the integration of biochemical signalling gradients and mechanical cues within XG-MA support baths to further mimic *in vivo* microenvironments. Additionally, adapting this platform for

co-culture systems or region-specific printing strategies may enable the fabrication of more complex musculoskeletal tissues, such as meniscus and/or osteochondral grafts.

Chapter V. 4D Bioprinting of Microtissues Within Temporally Adapting Support Baths to Engineer Anisotropic Musculoskeletal Tissues

The author of this thesis was the primary contributor to this chapter/published paper and was responsible for the design of the study, execution of all experimental work, data analysis, and preparation of the manuscript.

5.1 Introduction

The field of TE seeks to regenerate tissues or organs impaired by trauma or disease. Achieving this goal will require the engineering of regenerative grafts that replicate the complex structure, composition, and function of the native tissue. This is particularly true for the repair of musculoskeletal tissues, such as articular cartilage AC, meniscus, ligament and tendon, whose unique biomechanical function derives from the specialised architecture of their ECM. Damage to these tissues commonly initiates further joint degeneration, leading to the development of debilitating diseases such as osteoarthritis OA [331, 332]. Previous attempts at engineering such tissues have focused on seeding stem/progenitor cells within a 3D scaffold or a hydrogel, which are then stimulated in an attempt to promote tissue-specific ECM deposition [333-336]. However, these strategies typically fail to produce phenotypically defined tissues with biomimetic collagen network architectures that are integral to their physiological function, dramatically limiting their clinical utility.

Limitations associated with traditional TE strategies have motivated increased interest in scaffold-free approaches for the development of regenerative grafts. These strategies harness the ability of cells to self-organise in response to cell-cell and cell-matrix interactions, mimicking the self-organisation observed in polarised tissues during embryonic development and during adult tissue self-renewal [15]. Under specific physicochemical conditions, key aspects of such self-organisation can be replicated *in vitro*, allowing stem cells to generate μ Ts or organoids that mimic key features of a specific tissue or organ [337]. Under the appropriate conditions, it may be possible to combine such biological building blocks to engineer scaled-up tissue grafts [66, 134, 164, 172]. Realising

this goal will require the development of biofabrication strategies that can not only control the phenotype of the resulting graft but can also direct its (re)modelling to enable the engineering of tissues with user-defined ECM architectures.

During development, individual tissues almost never develop in isolation, but do so concurrently with surrounding tissues and organs, which mechanically confine, impinge upon, or pull on them [192, 338]. These mechanical influences have been postulated to affect the development of different tissues and, indeed, the entire early mouse embryo [253]. When cultured *in vitro* in the presence of physical boundaries, cells maintain the ability to sense and respond to such geometrical and physical cues [181]. For example, endothelial cell sheets exhibit geometry-dependent growth behaviour: on patterned substrates (i.e., squares or annular rings), proliferation is suppressed in the densely packed regions but persists at the periphery and in the mechanically stressed areas such as edges and corners. Similarly, the differentiation of adult MSCs is strongly influenced by geometric confinement and substrate stiffness [99, 339-341], with stiffer substrates and increased cytoskeletal tension promoting an osteoblastic phenotype [342]. These geometric and mechanical cues provide an additional layer of signalling, conveying positional information to distinct cells and guiding the maturation and organisation of tissues and organs [343]. Therefore, by providing spatially and temporally defined physical boundary conditions to populations of μ Ts or organoids, it may be possible to direct their fusion and (re)modelling in a programmable manner. If this can be achieved at scale, it would facilitate the transition of multiple individual μ Ts into scaled-up constructs that recapitulate the anatomical shape, internal structure, composition, and function of specific tissues and organs.

3D bioprinting is now an established technique to spatially pattern cells and supporting biomaterials to replicate key anatomical features of musculoskeletal tissues [259, 344] and engineer anisotropic musculoskeletal tissues. Such approaches have recently been extended to the bioprinting of cellular aggregates, μ Ts, and organoids [309, 345-347]. However, current bioprinting approaches often struggle to achieve precise μ T patterning and adequate fusion between μ Ts [235], which is a critical requirement for the engineering of scalable grafts. Moreover, little consideration has been given to how the bioprinting process can be used to direct long-term phenotype and structural (re)modelling of the resulting tissues. This could potentially be addressed using emerging 4D bioprinting concepts, such as the use of sacrificial or temporally adapting inks, but particularly the use

of support baths that temporally adapt to provide precise biophysical cues to printed cells [203, 348-352]. Previous studies have utilised such support baths to enable the high-resolution (bio)printing of low-viscosity (bio)inks [213], whereby print quality is determined by the physical properties (e.g. shear thinning, yield stress, stiffness) of the bath [233, 353]. For instance, the freeform reversible embedding of suspended hydrogels (FRESH) method employs extrusion into a gelatin slurry to support high-resolution 3D printing of a range of different inks [202, 315, 354], however, such support baths are typically designed to be removed post-printing and are not suitable for providing well defined physical constraints to bioprinted cells. This can potentially be addressed using baths that can be chemically crosslinked post-printing, thereby physically supporting the resulting construct during *in vitro* culture [355-357]. XG has previously been used as a support bath for bioprinting [211] and can be further functionalized using methacrylate to create a shear-thinning and self-healing photocrosslinkable bath [232]. The highly tunable nature of XG suggests that this material could be used as a support bath to not only facilitate the extrusion bioprinting of μ Ts or organoids, but also to modulate their physical environment post-printing.

Recognising that normal tissue development depends on both the self-organising potential of stem cells and key physicochemical cues from the microenvironment to establish its final architecture and function, here, temporally adapting methacrylate xanthan gum (XG-MA) support baths were used to control the surrounding matrix stiffness and the degree of physical confinement experienced by MSC-derived μ Ts post-printing. To this end, an extrusion-based bioprinting platform was used to precisely pattern μ Ts at a high density within a XG-MA support bath. By precisely tuning the physical properties (rheology, stiffness) of the support bath, we demonstrate that it is not only possible to modulate print fidelity, but also to direct μ T fusion, differentiation, and the long-term structural organisation of the bioprinted graft.

5.2 Materials and Methods

5.2.1 Modification of Xanthan Gum with methacrylic group (1H-NMR) and support bath preparation

XG (Sigma) was dissolved at 0.5% (w/v) in ultra-pure water and stirred until completely dissolved. Once dissolved, glycidyl methacrylate (40 mL) was added to the XG solution and stirred overnight at 60 °C, protected from light. The solution was now dialysed for 7 days against DI with water changes twice a day, freeze-dried for 48 hours, and stored at -20 °C for long-term storage. To ensure sterilization, aliquots of XG-MA were weighted and placed into a 50 mL tube and sterilized in an ethylene oxide gas sterilizer (Andersen Products) for 12 hours. 2 days prior printing, the material was dissolved into phenol free DMEM (Gibco) supplemented with penicillin (100 U mL⁻¹), streptomycin (100 µg mL⁻¹) (P/S) (both Gibco) and let it rotate at 40 rpm at RT for 2 days. The day of printing, Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (Sigma), was added for a final concentration of 0.25% (w/v) into the XG-MA solution and vortexed. To ensure the removal of the bubbles, the bath was centrifuged at 2500 x g for 5 minutes at RT, before printing. For each print, XG-MA (4 mL) was pipetted into a 6-well plate with a pipette for viscous liquids (Microman E, Gilson) to ensure no formation of bubbles during the pipetting.

5.2.2 Rheological assessment of bioink and support baths

Rheological characterisations were conducted on an MCR 102 Rheometer (Anton-Paar, Hertford Herts, UK) 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 (0.1–1000 s⁻¹) was measured at a constant temperature of 4 °C for the gelatin (Gelatin Type B) (Sigma) bioink, and at RT for the XG-MA support bath. To investigate the linear viscoelastic region (LVR) and determine the yield stress, an amplitude sweep test was carried out at RT. An angular frequency between 1 and 100 rad s⁻¹ was applied to the sample to determine G' and G'' curves. The G' and G'' crossover point was taken as the yield point where the material behaviour transitions from solid to liquid-like behaviour [358]. To evaluate the shear strain range (1-100%) and determine the solid and liquid-like state of the different inks, we performed a dynamic sweep stress test. Five intervals at a constant shear rate were performed to determine the thixotropy behaviour

of the support bath and investigate the time dependency of the support bath. Here, the viscosity was measured in transient shear rate step tests at RT. First, samples were sheared at a shear rate of 0.03 s^{-1} for 30 s, then the shear rate increased to 10 s^{-1} for 30 s, and again, the shear rate decreased to 0.03 s^{-1} for 30 s. Bioinks and support baths were maintained under high-humidity conditions to minimise dehydration during testing, and all rheological measurements were carried out in triplicate.

5.2.3 Compression testing of XG-MA hydrogels

Unconfined compression tests were performed on cylindrical samples prepared using a 5 mm PDMS mould and crosslinked under UV light for 4 minutes. After demolding, samples were immersed in PBS to prevent dehydration and subsequently compressed at a rate of $5\text{ mm}\cdot\text{s}^{-1}$ using a twin-column Zwick universal testing machine (Zwick Roell) equipped with a 10 N load cell. A preload of 0.01 N was applied to the hydrogels before testing. Compression was continued until sample failure, and the Young's modulus was calculated as the slope of the linear region of the stress–strain curve up to 70% strain.

5.2.4 Isolation and expansion of caprine BM-MSCs

Caprine bone marrow mesenchymal stem cells (MSCs) were isolated from the sternum of skeletally mature female goats as previously described [134]. Briefly, bone marrow was cut into smaller fragments and vortexed in high-glucose DMEM (hgDMEM, Gibco) supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS, Gibco), hereafter referred to as expansion medium (XPAN), to release cellular components. At ~80% confluency, cells were cryopreserved in liquid nitrogen for long-term storage. For further expansion, MSCs were reseeded at a density of $5 \times 10^3\text{ cells}\cdot\text{cm}^{-2}$ under 5% O_2 and expanded up to passage 3 (P3) for all bioprinting experiments.

5.2.5 Fabrication of MSC μ Ts

The μ Ts were fabricated as previously described [275]. Briefly, 3D-printed mould stamps containing 1,889 micro-resections and 4% (w/v) ultrapure agarose (Sigma) were sterilised in an autoclave at $120\text{ }^\circ\text{C}$ for 20 minutes. The agarose was then poured into each well of a 6-well plate, moulded with the stamps, and allowed to cool for 15 minutes. The resulting agarose microwell arrays were soaked overnight in XPAN at $37\text{ }^\circ\text{C}$ under physioxic conditions

(5% O₂, humidified atmosphere). MSCs were seeded into the microwells at a density of 7.556×10^5 cells·mL⁻¹ (5 mL per well), yielding 1,889 μ Ts with approximately 2,000 cells per μ T, and centrifuged at 700 $\times$ g to collect cells at the bottom of each well. The μ Ts were then cultured in chondrogenic medium (CDM) consisting of hgDMEM GlutaMAX supplemented with penicillin/streptomycin, sodium pyruvate (100 μ g·mL⁻¹), L-proline (40 μ g·mL⁻¹), L-ascorbic acid-2-phosphate (50 μ g·mL⁻¹), linoleic acid (4.7 μ g·mL⁻¹), bovine serum albumin (1.5 mg·mL⁻¹), 1 $\times$ insulin–transferrin–selenium (ITS), dexamethasone (100 nM) (all Sigma), and human transforming growth factor- β 3 (TGF- β 3, 10 ng·mL⁻¹; Peprotech, UK). After 2 days of culture, μ Ts were harvested for bioprinting. Media was gently flushed over the agarose surface with a micropipette and collected into a 50 mL tube, as previously described [134]. The density of the μ T suspension was quantified, and the required volume was adjusted to achieve the desired concentration for each bioprinting experiment.

5.2.6 Bioink preparation and bioprinting parameters

Sterile gelatin type B (1% w/v) was dissolved in hgDMEM for 30 minutes at 37 °C in a water bath the day prior to printing. After 30 minutes, gelatin is removed from the water bath and placed at 4 °C overnight for gelation. μ Ts are harvested from the agarose, placed in a 50 mL tube with media, and the tube is centrifuged at 650 $\times$ g for 5 minutes at RT to collect all the μ Ts at the bottom of the tube. Gelatin was then added to reach the desired μ Ts density for bioprinting. Afterwards, the bioink is carefully loaded into the cartridge and placed into the fridge for 15 minutes prior to bioprinting, in sterile conditions. The bioprinting is carried out using a syringe pump printhead (Cellink, Sweden), with an extrusion rate of 6 μ L s⁻¹ and print speed of 2 mm s⁻¹. Post-printing, the XG-MA support bath was UV-cured under sterile conditions using a 395 nm lamp (10 mW cm⁻²) for 4 minutes.

5.2.7 Live/Dead assay

For all the sample analysis, constructs were retrieved by mechanically disrupting the surrounding support bath and carefully extracting the samples using tweezers. Cell viability was assessed using a live/dead assay kit (Invitrogen). Briefly, constructs were rinsed with PBS, incubated for 1 hour at 37 °C in a staining solution containing calcein (2 μ M, excitation 485 nm, emission 530 nm) and Ethidium Homodimer (4 μ M, excitation 530 nm, emission 645 nm), and then rinsed again in PBS to remove excess dye. Samples were imaged with a

Leica SP8 confocal microscope, and maximum projection z-stacks were generated to evaluate viability throughout the tissue depth.

5.2.8 Scanning Electron Microscopy (SEM) imaging and analysis

Samples were fixed in 0.1M cacodylate buffer (Sigma) at 4 °C for a minimum of 12 h. The samples were enzymatically treated according to methods described before [89] to remove any excess of sGAGs that could disrupt the imaging of the collagen fibres. They were then washed with 1X PBS and dehydrated in a graded ethanol bath series (50-100% EtOH) and dried using the critical point drying method to better preserve the surface structure of the biological sample. Samples were imaged using a Zeiss Ultra Plus.

5.2.9 Histological Evaluation

Briefly, samples were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. After fixation, they were dehydrated through a graded ethanol series (50–100% v/v), cleared in xylene, and embedded in paraffin wax (Sigma). Sections of 5 µm thickness were cut, rehydrated, and subjected to histological staining. Haematoxylin and eosin (H&E; Sigma) was used to assess tissue morphology, ECM, and nuclei. Sulfated glycosaminoglycan (sGAG) content was stained with 1% (w/v) Alcian Blue (AB) 8GX in 0.1 M HCl and counterstained with 0.1% (w/v) nuclear fast red for cell distribution. Collagen deposition was visualised using 0.1% (w/v) Picrosirius Red (PR; Sigma). Stained sections were mounted in Pertex (Avantor) and imaged with an Aperio ScanScope slide scanner (Leica, Germany).

5.2.10 Immunofluorescence staining and quantification

Immunohistochemistry was performed for collagen type I (Abcam ab90395, 1:400), collagen type II (Santa Cruz sc52658, 1:400), for α -SMA (Abcam ab124964, 1:200) and YAP (Abcam ab3472, 1:200). Wax-embedded sections were rehydrated, washed with PBS, and, for α -SMA and YAP, subjected to antigen retrieval in sodium citrate buffer (10 mM, 0.05% Tween-20, pH 6) at 80 °C for 20 min. Samples were blocked with 10% donkey serum in PBS + 0.05% Tween-20, incubated overnight at 4 °C with primary antibodies, washed, and then incubated with secondary antibodies (1:200) for 1 h at room temperature in the dark. Sections were mounted with Fluoroshield containing DAPI (Sigma), sealed with nail polish, and stored at 4 °C until imaging. Fluorescence intensity was quantified from three ROIs per sample (n = 3) using ImageJ and normalised to the number of nuclei.

5.2.11 RNA isolation and quantitative Real-Time PCR

At 7 and 28 days after μ T bioprinting, samples were washed three times with PBS and snap-frozen for further processing. RNA was extracted using the Trizol (Sigma) method according to the manufacturer's instructions [11]. After chloroform extraction, RNA was resuspended in RNase-free water (Gibco) and stored at -80°C . cDNA was synthesised from 500 ng of RNA using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™) in a 20 μL reaction (37°C for 1 h, 95°C for 5 min, 4°C for 5 min). Real-time PCR was performed on an Applied Biosystems instrument using TaqMan Fast Advanced Master Mix (ThermoFisher). Goat-specific TaqMan probes (ThermoFisher) were used for target gene amplification, and 18S rRNA served as the housekeeping gene. The qPCR cycling program was: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 60°C for 1 min. Gene expression was quantified using the $2^{-\Delta\Delta\text{Ct}}$ method, normalised to 18S rRNA levels, and compared with MSC monolayer controls.

5.2.12 Filament diameter measurements and polarised light microscopy analysis

The diameter of bioprinted μ Ts was measured using an inverted microscope (Primo Vert, Zeiss, USA) with a 4 $\times$ objective. Images were acquired at defined time points up to 28 days post-printing, and diameters were quantified with ImageJ software (Wayne Rasband and contributors, USA). For each replicate, four independent measurements were obtained per construct. For polarised light microscopy (PLM), paraffin-embedded tissue sections were stained with 0.1% (w/v) Picrosirius Red (Sigma-Aldrich), mounted with Pertex (Sigma-Aldrich), and imaged using an Olympus BX41 polarising microscope equipped with a MicroPublisher 6™ CCD camera and a U-CMAD3 adaptor. Collagen fibril orientation, dispersion, and coherency were analysed using the OrientationJ and Directionality plugins in ImageJ.

5.2.13 Statistical analysis and graphical results

Statistical analyses were performed using the software package GraphPad Prism (version 10.4.1). All data were pre-processed before analysis. Outliers were identified and excluded using the ROUT method where applicable. Statistical tests used to assess statistical differences between groups are indicated in the Fig. legends. For experiments involving comparison of more than two groups under a single experimental factor, one-way ANOVA

followed by Tukey's post hoc test was applied. For experiments involving two variables, two-way ANOVA followed by Tukey's post hoc test was used to evaluate interaction effects and main effects. When groups were compared, significance was accepted at a level of $p < 0.05$. Statistical significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Results are expressed as mean $\pm$ standard deviation. The sample size (n) for each experiment is reported in the corresponding Fig. legends were produced with GraphPad Prism.

5.3 Results

5.3.1 Development of a temporally adapting support bath for extrusion bioprinting of μ Ts

To control the spatial deposition of μ Ts within the XG-MA support bath, a bioink compatible with extrusion bioprinting was required. This bioink needed to permit smooth extrusion and precise patterning of highly concentrated μ Ts, and subsequently clear rapidly from the printed channels (following UV crosslinking of the bath) to enable μ T fusion and self-organisation directed by the physical boundaries formed during printing. To determine whether the gelatin would vacate the printed channel by diffusing into the support bath, we performed a perfusion test using black ink after printing the gelatin ink (Fig. S 5.1). Within 15 seconds, the ink was able to flow through the void channel left by the liquefied gelatin, confirming its sacrificial behaviour when printed into the support bath. A 1% gelatin solution was selected as the bioink that best fulfilled these criteria, as it undergoes gelation at low temperature during printing while exhibiting favourable shear-thinning behaviour for extrusion. Accordingly, μ Ts were extruded into the support bath encapsulated in 1% gelatin at a density of 45,000 μ Ts/mL, previously identified (Chapter IV) as the optimal concentration for both smooth extrusion and effective post-printing fusion (Fig. 5.1A).

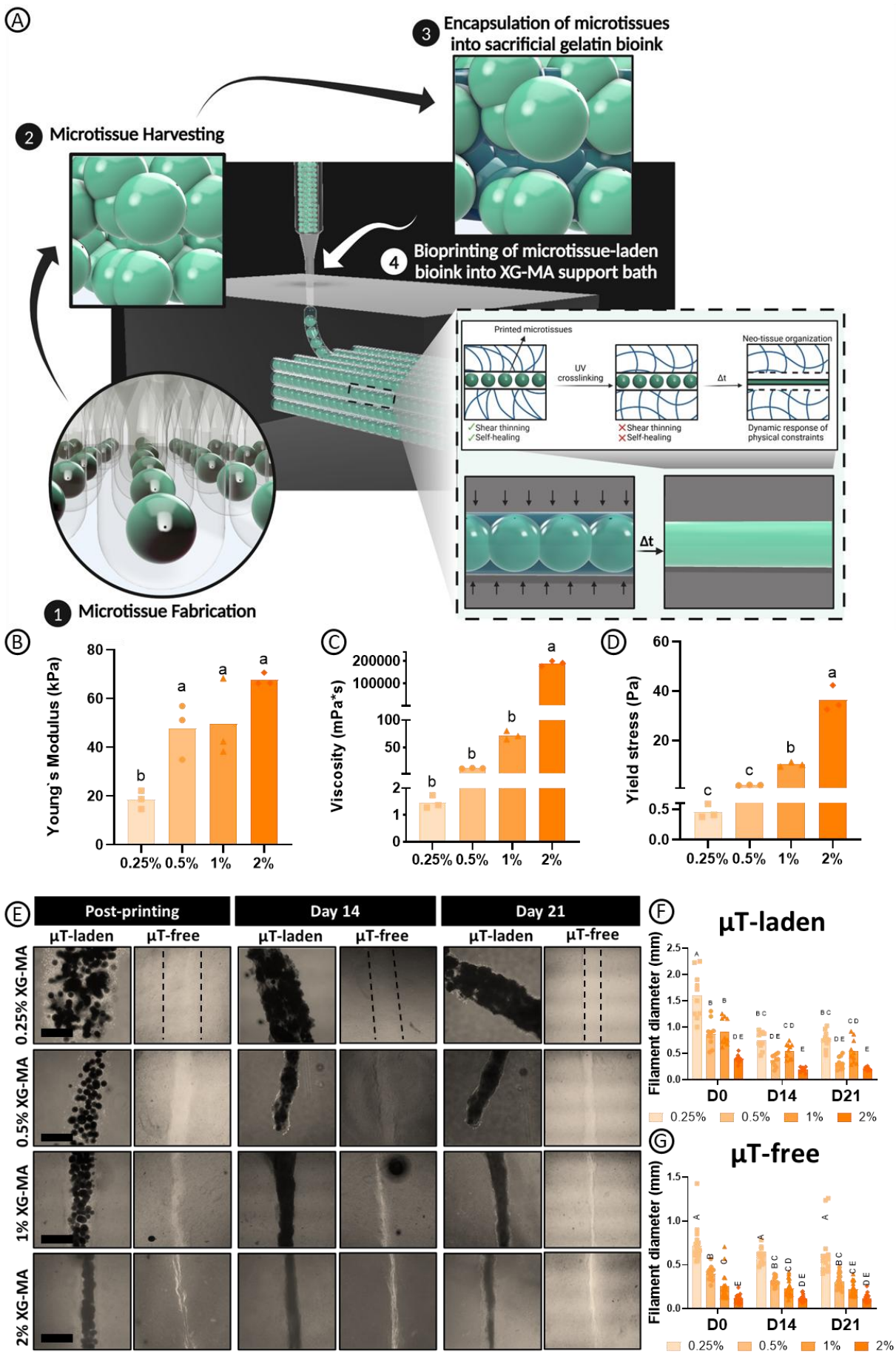


Figure 5.1. The concentration of the support bath influences the extent of filament confinement over time post-printing. A) Graphical abstract of the 4D-bioprinting platform. μ Ts are fabricated using a high throughput agarose microwell system and cultured for 2 days prior to their harvest and encapsulation into a gelatin bioink and extrusion into a XG-MA support bath. After UV-crosslinking, the filament diameter changes over time due to the changing physical confinement provided by the support bath. B) Young's Modulus of XG-MA hydrogels following UV crosslinking for 4 minutes. C) Viscosity of the un-crosslinked XG-MA bath at different concentrations. D) Yield stress of the un-crosslinked XG-MA bath. E) Brightfield microscope imaging of the printed μ Ts within XG-MA support baths of differing concentrations at different time points. Filament diameter changes over time for the (F) μ T-laden inks and the (G) μ T-free inks. Scale bar (Sb): 200 μ m. 2-way ANOVA followed by the Tukey's post comparison test was performed to assess the differences between the groups at different time points; significance was accepted when $p < 0.05$. Statistically comparable groups are labeled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

To enable the high-resolution bioprinting of μ Ts, we next sought to extrude them into a support bath with tailorable mechanical and rheological properties. We selected XG as a support bath and further modified it with a methacrylate group (XG-MA) to stabilise and mechanically support the printed filament throughout the culture process (Fig. 4.5). 1.5% XG or 1% XG-MA have previously been used as a support bath for bioprinting [211, 232]. To expand on this, we tested a range of XG-MA concentrations (from 0.25 to 2% w/v) and measured their stiffness post UV crosslinking. We found that the Young's modulus increased with XG-MA concentration, from 20 kPa for the softest bath to 65 kPa for the stiffest bath (** $p < 0.01$; Fig. 5.1B). Stiffness of the support baths was assessed over time (Fig. S5.2A), with different concentrations tested at day 0 and day 7 under physiological conditions. Although the Young's modulus decreased across all groups, differences in stiffness between concentrations were maintained after 7 days (Fig. S5.2A). All tested concentrations of XG-MA displayed shear-thinning and thixotropic properties, allowing the bath to maintain its structural integrity post-printing (Fig. S5.2 D-F). Irrespective of concentration, the XG-MA was able to recover after experiencing high shear stress, with a faster recovery observed in the higher concentration baths (Fig. S 5.3C). Having developed a support bath with tunable mechanical and rheological properties, we next sought to assess its capacity to support high-resolution extrusion bioprinting of high-density μ Ts-laden bioinks. Using the same extrusion printing parameters (6 μ L s^{-1} extrusion rate, 2 mm s^{-1} printing speed), a higher concentration XG-MA support bath enabled a higher printing

fidelity/resolution (i.e. a smaller printed filament diameter) for both a μ T-free and a μ T-laden ink (Fig. 5.1 F, G). The printed μ Ts generated a larger diameter filament when printed into a softer bath, ranging from 1.5-2mm in diameter following extrusion into a low stiffness 0.25% bath, to 0.25-0.5 mm in the stiffer 2% bath for both μ T-laden (Fig. 5.1F) and μ T-free inks (Fig. 5.1G). To assess the smoothness of printed microtissue filaments, variations in filament diameter were quantified from brightfield microscopy images (Figure S 5.3A). For each filament, five measurements were taken along its length, and the standard deviation of these values was used as an index of smoothness. Lower standard deviations reflect more uniform diameters (smoother filaments), while higher values indicate greater irregularity (Figure S 5.3B). As fusion progressed, this standard deviation decreased, consistent with increased tissue compaction. Notably, even immediately after printing, filaments in the stiffer support baths (1% and 2% XG-MA) displayed lower standard deviations, indicating higher initial smoothness (Fig. S 5.3B). Therefore, the physical properties of the bath contribute to filament compaction and smoothness, complementing the filament diameter analysis. 7 days post-printing, the μ Ts appeared to have fused, with high cell viability observed in all support baths (Fig. S5.4 A). This suggests that the shear stress applied to the cells within the μ Ts during the bioprinting process, as well as UV exposure to crosslink the support bath post-printing, does not negatively impact cell viability.

The concentration of XG-MA in the support bath influences the initial diameter of the printed filament but also impacts how it changes with time in culture. In μ T-laden bioinks, the filament diameter was found to reduce in diameter with time in culture, with larger absolute reductions observed in the softer baths (Fig. 5.1 F, G). However, even in the μ T-free prints (where no cellular remodelling is involved), a reduction in filament diameter over time is observed, pointing to an active role for the support bath in determining the long-term geometry of the print (Fig. 5.1 F, G). Collectively, these results suggest that the support bath exerts a radially compressive force on the filament post-printing, resulting in a temporal reduction in diameter until an equilibrium is reached between day 14 and 21 (Fig. 5.1 G).

5.3.2 YAP activation in MSC-derived μ Ts is influenced by support bath stiffness

As the stiffness of the support bath increased with XG-MA concentration (Fig 5.1B), we next investigated whether such differences in the local mechanical environment would influence the activity of known mechanosensitive proteins in the bioprinted μ Ts (Fig. 5.2). Here, bioprinted μ Ts were maintained in media supplemented with TGF- β 3 post-printing. To assess YAP activity across different bioprinting conditions, we performed YAP staining at both early and late time points in constructs printed into XG-MA support baths of differing stiffness (Fig. 5.2 A). YAP activity was significantly higher in μ Ts printed into the stiffer support baths (1% and 2% XG-MA). In these stiffer conditions, greater YAP nuclear colocalization was also observed (Fig. 5.2 A, green inserts). This suggests that the greater stiffness of higher concentration XG-MA baths promotes YAP transcription and its activation.

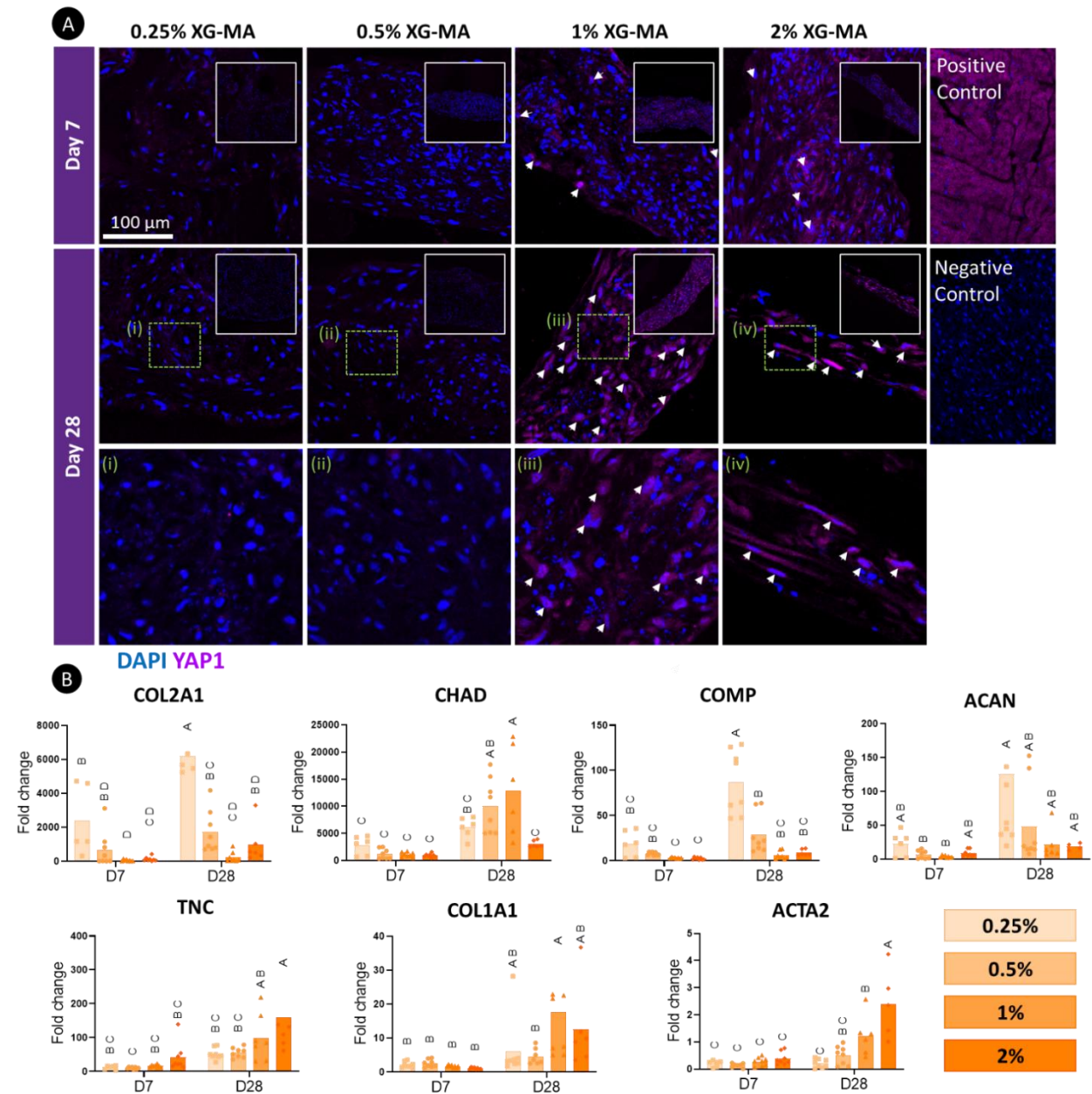


Figure 5.2. The physical properties of the support bath influence MSCs' phenotype. A) YAP1 staining of printed μ Ts reveals spatial differences in YAP expression depending on the stiffness of the support bath. White arrows indicate regions where YAP colocalises with the nuclei, indicating YAP activation. Positive control is the mouse heart, and negative control is the mouse heart without the primary antibody. B) Expression of a range of genes associated with cartilage (COL2A1, CHAD, COMP, ACAN), fibrocartilage or ligament tissue (COL1A1, TNC, ACTA2). For statistical analysis, a two-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. Statistically comparable groups are labelled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

5.3.3 Support bath stiffness regulates μ T phenotype

As YAP activity within the bioprinted μ Ts was strongly regulated by the stiffness of the surrounding support bath, we further hypothesised that longer-term MSC phenotype would also depend on these physical cues. To this end, the printed constructs and the MSC phenotype were assessed over a 4-week culture period using a range of biochemical and histological assays. We first assessed the expression of a range of genes associated with chondrocytes, fibrochondrocytes, and myofibroblasts (Fig. 5.2B). The highest levels of ACTA2 expression (a gene that encodes for smooth muscle alpha-2 actin) were observed in the stiffer 2% baths after 28 days of culture (Fig. 5.2C).

We also assessed α -SMA deposition through immunofluorescence staining (Fig. 5.3 A). While weak α -SMA staining was observed at day 7 (Fig. 5.3A), a significant increase was evident by day 28, particularly in the stiffer (1% and 2%) baths ($p < 0.05$) (Fig. 5.3 A, B). COL1A1 and TNC (Tenascin-C) gene expression, markers associated with a ligamentous or tendon phenotype, was also higher in the stiffer baths (Fig. 5.2 C). In agreement, type I collagen deposition was higher in the stiffer support baths at both early and late time points (Fig. 5.3 A, C).

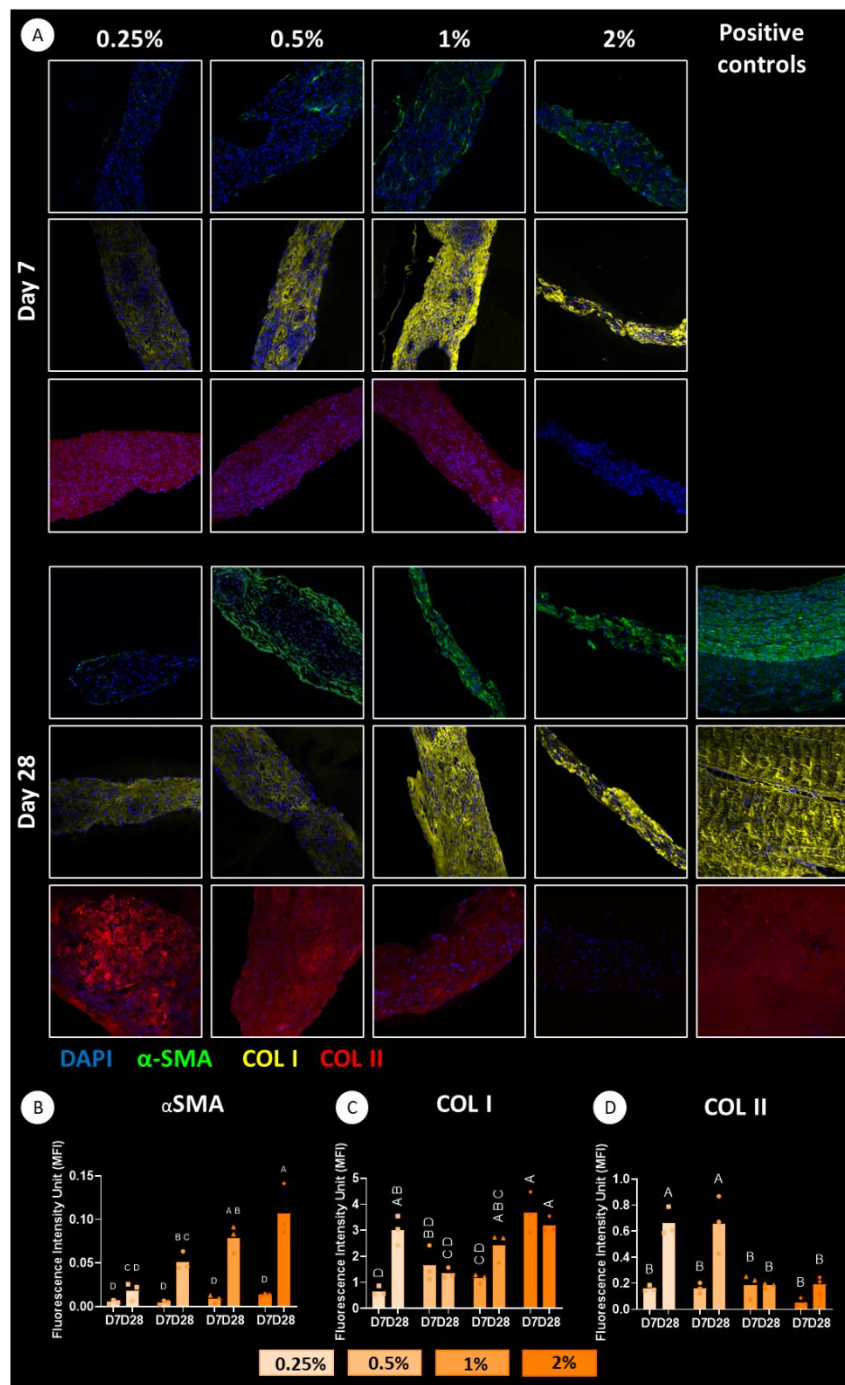


Figure 5.3. Immunofluorescence staining for specific proteins within the bioprinted μ Ts. (A) Staining for type I collagen, type II collagen and α -SMA deposition in 7- and 28-day post-printing in the different support baths. Positive controls are arterial control for α -SMA, ligament for type I collagen and articular cartilage for type II collagen. Quantification of immunofluorescence intensity normalised with background intensity and normalised to the number of nuclei of each region of interest (ROI) for α SMA (B), collagen I (C), collagen II (D); n=3. For all the graphs, a two-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. Statistically comparable groups are labelled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

While genes and proteins associated with a ligament or tendon phenotype were more highly expressed in the stiffer bath, the expression of the chondrogenic genes COL2A1, COMP and ACAN was higher in μ Ts bioprinted into the softer support baths (Fig. 5.2 B). In agreement with this finding, strong staining for type II collagen deposition was observed in the tissues generated in the soft baths, with little deposition observed in the stiffer baths (Fig. 5.3 A, D). The softer 0.25% and 0.5% XG-MA support baths promote greater collagen II deposition and reduced ACTA2 expression, resulting in a more chondrogenic phenotype (Fig. 5.3). The observation of enhanced sGAG accumulation in these softer environments further confirms this conclusion (Fig. S 5.4 B). The 1% XG-MA bath, which possesses an intermediate stiffness, appeared to support a more fibrochondrogenic phenotype, as evident by simultaneous sGAG (Fig. S 5.4B) and type I collagen deposition (Fig. 5.3 A, C). Finally, the stiffest 2% XG-MA baths supported the highest levels of overall collagen (Fig. S5.4 C) and specifically type I collagen production (Fig. 5.3 A, B) but little sGAG deposition (Fig. S 5.4 B, C), suggesting the development of a more ligamentous tissue.

5.3.4 Support bath stiffness regulates collagen fibre alignment in bioprinted tissues

Encouraged by the successful bioprinting of high-density μ T-laden bioinks into a temporally adapting support bath, I next sought to explore if this bioprinting platform could be used to direct collagen organisation and hence enable the engineering of anisotropic soft tissues. To validate the ability to bioprint custom-shaped, highly aligned musculoskeletal tissues, PLM image analysis was employed to assess the preferential alignment of collagen fibres along the printed filaments (Fig. 5.4).

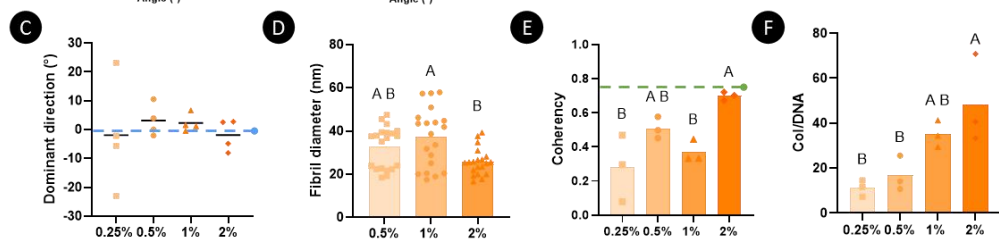
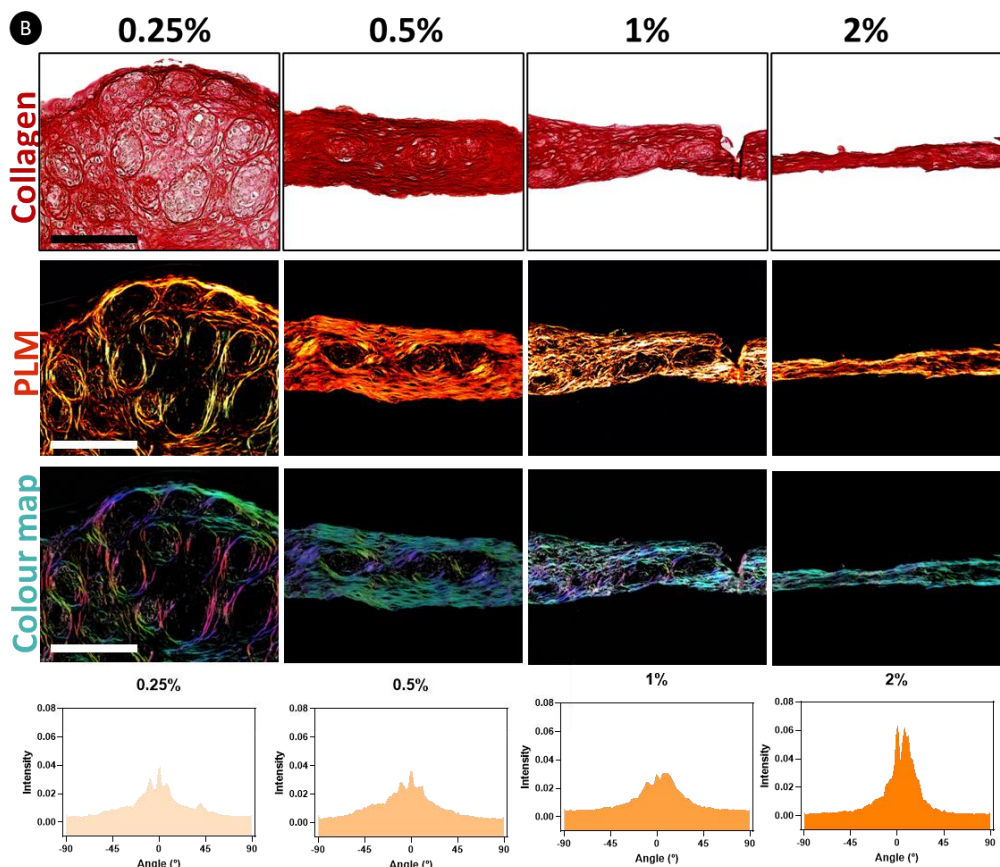
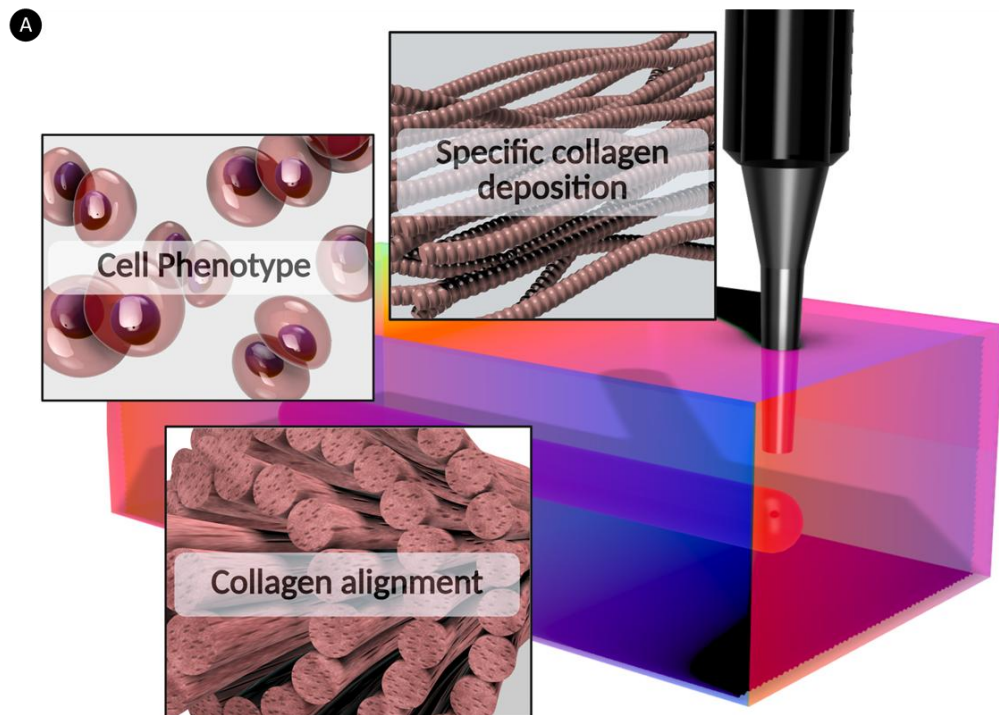


Figure 5.4. A stiffer support bath improves μ T fusion and the alignment of the secreted collagen fibres. A)

Graphical abstract of a 4D bioprinting platform capable of generating phenotypically distinct musculoskeletal grafts. B) Histological assessment of bioprinted μ Ts after 28 days post-printing with picosirius red staining, using PLM imaging to analyse collagen fibre alignment. Colour maps are generated from PLM images. Colour hue represents fibre orientation, with blue/cyan corresponding to fibres aligned at 0° and pink/red corresponding to fibres aligned at 90°. Sb: 200 μ m. Quantification of the fibre orientation was then performed for the 0.25%, 0.5%, 1% and 2% XG-MA support baths. C) Average collagen fibre orientation with the filaments generated in the different support baths. D) Fibril diameter at 28 days post-printing. E) Fibre coherency was quantified, with values approaching 1 indicating highly aligned fibres and values closer to 0 reflecting greater dispersion in multiple directions (n = 4). F) Biochemical quantification of total collagen normalised to DNA levels. Statistical difference is determined using a two-way ANOVA test with Tukey's post hoc comparison test. Significance was accepted with $p < 0.05$. Statistically comparable groups are labelled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

After four weeks of *in vitro* culture, the μ Ts fused to generate filaments of tissue whose internal structure was directed by the physical constraints imposed by the XG-MA support bath. PR staining (Fig. 5.4 A) revealed robust collagen deposition across all groups, with more homogeneous collagen distribution observed within filaments printed in the stiffer baths (Fig. 5.4 A). Collagen deposition, quantified relative to DNA levels (Fig. 5.4 E), was also significantly higher in the stiffer baths ($p < 0.01$). In addition, the XG-MA bath provided effective boundary conditions for directing collagen organisation in the neotissue. While collagen fibres tended to align parallel to the print direction (i.e. at 0 °C) irrespective of the bath stiffness (Fig. 5.4 B), the stiffer 2% support bath supported the greatest degree of fibre alignment (Fig. 5.4 A) and coherency (Fig. 5.4 D). We also investigated the size of the collagen fibrils through SEM imaging (Fig. S 5.5), where preferential alignment of the collagen fibres was observed on the surface of all groups. The diameter of the collagen fibril ranged between 20 and 60 nm (Fig. 5.4 C) after 4 weeks of *in vitro* culture, which is comparable to that observed in the cartilage of skeletally immature pigs [89].

5.3.5. Bioprinting of anisotropic musculoskeletal tissues by spatial patterning μ Ts into support baths to differing stiffness

To demonstrate the utility of this μ T-based bioprinting platform, I next sought to engineer larger tissue grafts with distinct phenotypes and anisotropic collagen fibre networks (Fig. 5.5). As a proof of concept, I sought to replicate the distinct collagen arrangements observed in different musculoskeletal tissues, specifically the arcade-like collagen alignment in AC [359, 360], circumferentially aligned fibres in the menisci [361], and the linear collagen orientation found in ligaments [362]. To maintain anisotropy across multiple layers of printed μ T filaments, I optimised filament spacing within the support bath to promote tissue compaction while preserving the desired collagen organisation (Fig. S5.6). For all of these prints, the composition of the culture media remained constant, with only the stiffness of the bath changing for the bioprinting of the different tissue types.

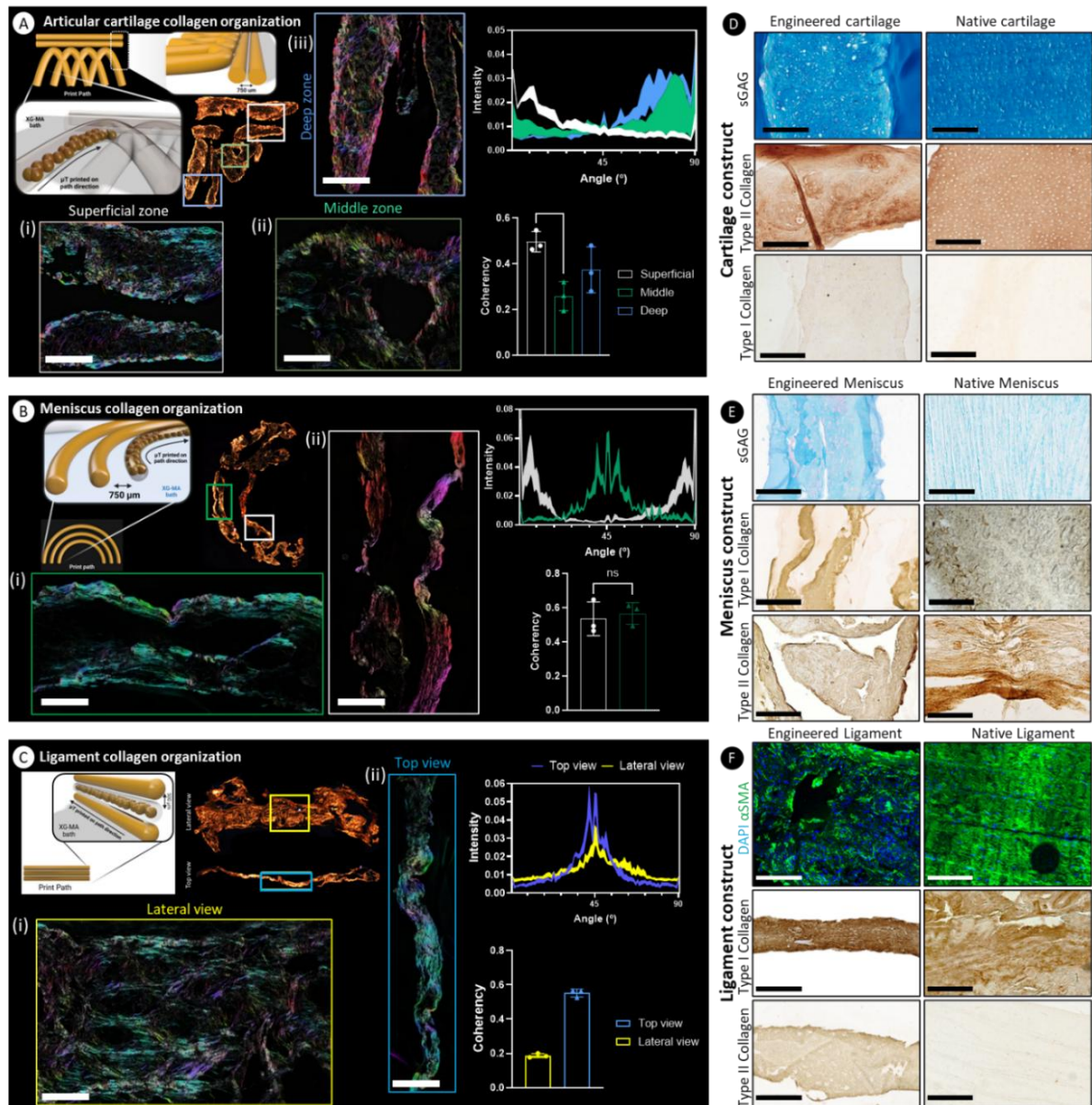


Figure 5.5. The bioprinting of scaled-up anisotropic tissue grafts. A) Bioprinting of Benninghoff collagen arcades is typical of native AC. The collagen fibre orientation was quantified in the superficial (i), middle (ii) and deep (iii) regions of the tissue, indicating the development of highly anisotropic tissues. Coherency analysis values closer to 1 indicate that the fibres are aligned in one direction, while values close to 0 indicate the fibres are aligned in different directions. White indicates the colour map of the superficial zone, green indicates the colour map of the middle zone, and blue indicates the colour map of the deep zone. Scale bar: 200 μ m. B) Bioprinting of circumferential filaments to engineer a meniscal tissue. Images from two different regions (green (i) and white (ii) boxes) are provided, again demonstrating that the collagen fibres are aligning according to the printing path. Fibre coherency analysis indicates that most of the fibres are aligned in one direction. The green lines/bars represent the collagen fibre orientation within the regions highlighted within the green boxes (i). The white lines/bars represent the collagen fibre orientation within the regions highlighted within the white box (ii). Scale bar: 200 μ m. C) Bioprinting of a 3-layered anisotropic ligament

tissue, where most of the fibres are aligned towards the 0 °C direction in both the lateral (yellow colour box (i)) and top (blue colour box (ii)) view. Scale bar: 200 µm. A one-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. *Indicates $p < 0.05$. ns: non-significant. For all the graphs: $n = 3$. D) Histological and immunohistochemical assessment for the cartilage construct for sGAG, type II collagen, and type I collagen deposition compared to native AC. E) Histological and immuno-histological assessment for the meniscal construct for sGAG, type I collagen, and type II collagen, compared to the native menisci. F) Histological and immunohistochemical assessment for the ligament construct for α -SMA, type I collagen, and type II collagen compared to the native ligament. Scale bars: 200µm for type I and II collagen and 100 µm for α -SMA.

The μ Ts were first extruded into a 0.5% XG-MA support bath to generate an arcade-like geometry characteristic of native AC (Fig. S 5.7A), where they fused and reorganised over time, aligning according to the print path, and generating a cartilaginous tissue rich in type II collagen (Fig. 5.5 D). In the superficial zone of the resultant tissue, collagen fibres predominantly aligned parallel (0 °C) to the surface of the construct, resembling that observed in native AC (Fig. 5.5 A (i, iv)). In the deep zone, fibres were primarily oriented at 90 °C, reflecting the perpendicular alignment seen in native AC (Fig. 5.5(iii, iv)). PLM quantification confirmed the superior tissue organisation resulting from the support bath's temporal confinement, leading to effective μ T fusion and ECM reorganisation (Fig. 5.5 A). Furthermore, the soft XG-MA support bath supported a chondrogenic phenotype, characterised by robust deposition of the hyaline cartilage-specific matrix markers sGAGs and type II collagen (Fig. 5.5 D). For the meniscus construct, μ Ts were bioprinted in the intermediate stiffness 1% XG-MA support baths in a circumferentially aligned pattern to replicate the organisation seen in the meniscal ECM (Fig. S 5.7B). PLM analysis confirmed that the final construct displayed high collagen fibre anisotropy, with fibre orientation closely matching the printing direction (Fig. 5.5 B). The meniscus is composed of two distinct regions: the inner zone, which contains a mix of collagen types I and II, and the outer zone, primarily made up of collagen type I. Here, I successfully engineered a highly anisotropic meniscal tissue with uniform sGAG deposition and a high type I collagen content (Fig. 5.5 E)—characteristics typical of the outer zone, which accounts for over 80% of the meniscus body [363]. As a final proof of principle, I attempted to bioprint a ligament-like tissue within the stiffer 2% XG-MA support bath (Fig. 5.5 C, F) (Fig. S5.7C). To this end, I bioprinted a three-layered structure with parallel collagen alignment to replicate the

ligament architecture (Fig. 5.5 C). Here, MSC-derived μ Ts adapted to their surroundings, secreting type I collagen and expressing α -SMA (Fig. 5.5 F), indicating (myo)fibroblastic differentiation. Therefore, the physical boundaries provided by the support bath, applied to μ T-rich bioinks, can be used to direct neo-tissue organisation and enable the engineering of phenotypically distinct musculoskeletal tissues.

5.4 Discussion

Bioprinting large numbers of μ Ts into a support bath required a printing strategy that could both stabilise the constructs during deposition and enable μ T fusion post-printing. Gelatin has previously been used as a sacrificial ink in support baths, enabling the 3D printing of perfusable channels for vascular TE [364, 365]. In this context, gelatin functioned effectively as a sacrificial carrier: it enabled smooth extrusion and precise patterning of μ Ts, yet subsequently dissolved and diffused away, leaving the μ Ts free to fuse. This transient role is particularly important in μ T-based printing, where excessive permanence of the bioink can act as a physical barrier and delay or even prevent the formation of a continuous tissue [66, 218]. Equally critical was the choice of μ T density within the gelatin. At low densities, printed filaments exhibit gaps and discontinuities, whereas very high densities lead to clogging and irregular deposition. Identifying an intermediate density that allowed continuous extrusion while promoting post-printing fusion is therefore essential for generating cohesive tissue filaments rather than fragmented constructs.

Beyond the sacrificial bioink, the properties of the surrounding support bath played a central role in defining both the initial and evolving architecture of the printed structures. Previous studies have typically focused on the printability of bioinks and on post-printing outcomes from a purely geometric perspective. Here we sought to combine a sacrificial bioink with a non-sacrificial support bath, thereby creating boundary conditions that change over time to direct cell–cell and cell–matrix interactions, ultimately regulating phenotype and structural organisation of resulting tissue. To generate a non-sacrificial support bath, it was essential to utilise a material that could be crosslinked post-printing, thereby stabilising the printed structure throughout the *in vitro* culture. This is in contrast with other commonly used support baths, which are designed to provide only temporary

physical support during the printing process and to rapidly degrade post-printing [202, 211, 315, 354]. By employing a photocrosslinkable XG-MA, the system combined the short-term advantages of embedded printing [200, 211, 232, 311] with longer-term mechanical support and confinement [228]. Increasing bath stiffness improved print fidelity and enabled the formation of thinner, smoother filaments, highlighting how the mechanical environment during and immediately after extrusion can be used to refine geometric control. Over time in culture, the diameter of μ T filaments progressively decreased, particularly in softer baths, reflecting a combination of cell-driven compaction, loss of the sacrificial gelatin bioink, and radially compressive forces exerted by the surrounding bath. Notably, reductions in filament diameter were also observed in μ T-free prints, underscoring that the support bath itself actively shapes the construct geometry rather than serving as a purely passive medium. Throughout this process, high cell viability was maintained, indicating that the imposed constraints and UV crosslinking conditions were compatible with long-term culture.

Viewed together, these findings position the XG-MA support bath as a platform for a form of 4D bioprinting, in which time-dependent changes are governed predominantly by the evolving physical boundaries around the printed μ Ts. In traditional 4D printing approaches, this dynamic transformation is typically achieved using materials that respond to environmental factors such as temperature, pH, humidity, etc. [261, 366-368]. With such approaches, temporal changes within the bioink (e.g., swelling, shrinking, or controlled degradation) support the dynamic transformation of the construct [369, 370]. For example, programming delayed dissolution into sacrificial bioinks has previously been used to provide precise control over the spatial and temporal introduction of architectural features into cell-laden hydrogels, which was leveraged to support the development of a pre-vascularized, bone-like graft [348]. Other approaches leverage cell-generated forces within bioinks to control shape-morphing in support baths post-printing [371]. In contrast, in our system, it is the support bath that primarily provides the dynamic component: the surrounding environment- the physical boundaries- evolves over time, guiding μ T fusion, compaction, and alignment of the printed filament. Previous studies have shown that such physical boundaries can guide neo-tissue organisation, enabling the engineering of anisotropic living systems [183, 372]. Furthermore, tissue maturation *in vivo* is driven by

spatiotemporal cellular signalling and mechanical forces [373]. Therefore, we hypothesised that the degree of physical confinement imposed by the bath over time would influence μ T fusion and phenotype, as well as the structural organisation of the resulting tissue, thereby enabling the bioprinting of anisotropic grafts.

MSCs are known to respond to the stiffness of their surrounding environment [374, 375]. It is well established that physical and biochemical signals work in synergy to guide cell fate and function [376, 377]. YAP activation is known to favour osteogenic programmes while antagonising adipogenic and chondrogenic differentiation [378-385], and all constructs in this study were exposed to TGF- β 3, a cue whose effects are strongly context dependent [280, 386]. Increasing the stiffness of the XG-MA bath elevated YAP activity and nuclear localisation, consistent with its role as a mechanotransducer downstream of actomyosin tension and ECM mechanics. As YAP activity within the bioprinted μ Ts was strongly regulated by the stiffness of the surrounding support bath, we further hypothesised that longer-term MSC phenotype would also depend on these physical cues. In the softer baths, the μ Ts tended to develop a more cartilage-like identity: they produced more cartilage-associated matrix and accumulated higher levels of proteoglycans, while markers linked to highly contractile, fibrous cells were relatively low. As stiffness increased, this balance progressively shifted. At intermediate stiffness, the tissues adopted a fibrocartilaginous phenotype. In the stiffest baths, the constructs instead resembled more ligament-like tissue, with abundant fibrous collagen and little evidence of a cartilage-type matrix. In this context, the support bath does not simply act as a passive printing aid but functions as an active mechanical microenvironment that shapes intracellular signalling. Therefore, under identical biochemical stimulation, mechanical tuning of the support bath can bias MSC fate and ECM remodelling within the printed μ Ts. Consequently, support bath design should be considered a key mechanobiological variable when engineering anisotropic, tissue-specific grafts, rather than being treated solely as a parameter for print fidelity.

The successful bioprinting of high-density μ Ts into a support bath that adapts over time, allowing fusion and phenotype modulation as a function of stiffness, raised the question of whether the same platform could also be used to control tissue architecture. Instead of relying on collagen-based bioinks, which are notoriously slow to gel and difficult to print with high fidelity [387-389], the strategy here was to let preformed μ Ts generate their own

ECM within a physically constrained environment. As the μ Ts fused over several weeks, the surrounding bath provided fixed boundaries that shaped how the newly deposited collagen was organised. Across conditions, this led to the formation of continuous tissue filaments in which collagen tended to align along the printing direction. Stiffer baths supported higher collagen deposition and an enhanced degree of collagen alignment along the print axis. In softer environments, collagen was still deposited and showed some preferential orientation, but the extent of alignment and overall collagen fibre coherency was reduced. Taken together, these findings indicate that the mechanically defined boundaries provided by the support bath can be harnessed to guide both the quantity and the organisation of collagen produced by the bioprinted μ Ts to engineer anisotropic tissue.

Musculoskeletal tissues, particularly those that endure mechanical loads in specific directions, exhibit preferential collagen fibre alignment to support their biomechanical functions. For example, tendons and ligaments have significantly higher tensile properties along the fibre direction [70, 250, 362]. In the meniscus, the predominance of circumferentially aligned type I collagen fibres provides the necessary stiffness (100–300 MPa) to enable this tissue to function within the knee joint [390]. Similarly, in AC, vertically oriented collagen fibrils in the deep zone protect the tissue against large compressive strains [359], while collagen fibres aligned parallel to the surface in the superficial zone play a crucial role in resisting high tensile stresses [360, 391]. With this in mind, the μ T-based bioprinting platform was next used not only to specify cell phenotype, but to reconstruct these distinct collagen organisations in a controlled way. In a softer environment, μ Ts printed in an arcade-like geometry developed a cartilage-like construct, with collagen aligned parallel to the surface in the outer region and perpendicular in deeper layers, closely echoing native AC architecture. This was accompanied by an ECM rich in cartilage-associated components, consistent with a chondrogenic phenotype. Using an intermediate stiffness and a circumferential print pattern, the same μ T building blocks assembled into a meniscus-like tissue with highly aligned collagen, uniform proteoglycan deposition, and a predominantly type I collagen matrix reminiscent of the outer region of the meniscus. In the stiffest bath, a simple parallel filament architecture yielded a ligament-like construct, where the μ Ts adopted a (myo)fibroblastic phenotype and produced abundant type I collagen aligned along the print direction. Collectively, our results highlight that the physical

boundaries provided by the support bath, applied to μ T-rich bioinks, can be used to direct neo-tissue organisation and enable the engineering of phenotypically distinct musculoskeletal tissues. A limitation of this work is that the individual tissue filaments do not fully fuse in the support bath, likely due to the persistence of the bath material between adjacent filaments, which may negatively impact the overall functionality of the bioprinted construct. Future work will explore the implementation of modified support baths that not only provide fusing μ Ts with sufficient mechanical support to direct their differentiation and remodelling but also degrade in a more programmable manner to ensure that residual bath material does not prevent the development of a cohesive, scaled-up graft. In turn, this will also allow us to assess if the proposed bioprinting platform can generate biomechanically functional tissues suitable for *in vivo* implantation.

5.5 Conclusion

In summary, this novel bioprinting platform, which leverages a temporally adapting support bath, creates a dynamic environment that enables the precise spatial patterning of μ T-laden bioinks. We demonstrate that the physical environment created by the surrounding support bath can modulate the phenotype of the printed μ Ts, enabling the engineering of different musculoskeletal tissues by simply varying the support bath stiffness. Remarkably, this approach not only supports distinct musculoskeletal phenotypes but also controls μ T (re)modelling and subsequent ECM organisation, thereby enabling the engineering of anisotropic grafts with user-defined collagen architectures. Furthermore, we demonstrate the versatility of this platform by bioprinting a range of different musculoskeletal tissues, including AC, meniscus, and ligament grafts. These findings support the continued development of 4D bioprinting strategies that more closely mimic normal developmental processes to better engineer functional tissues or organs. In the future, such bioprinting platforms could potentially provide implantable, functional grafts at scale for a range of different regenerative medicine applications.

Chapter VI: Refinement of a μ Ts bioprinting Platform

Incorporating a Degradable Support Bath

6.1 Introduction

3D bioprinting has traditionally relied on biomaterial inks to provide temporary structural support for printed cells. While these materials are effective in maintaining shape during fabrication, they can also impair cell viability and maturation due to excessive shrinkage, limited remodelling, or even immunogenic responses [19, 77, 137, 140, 219, 228, 329, 388]. In the broader field of TE, such drawbacks are being addressed using scaffold-free approaches where engineered constructs primarily depend on the cells themselves and their intrinsic ability to produce ECM [15]. Scaffold-free strategies rely on cellular “building blocks” such as sheets, strands, rings, aggregates, and μ Ts to overcome the challenges associated with non-sacrificial biomaterials and to enable the engineering of functional tissues [15]. These building blocks can fuse into larger constructs, producing mechanically more competent tissues as the cells secrete ECM and self-organise into stronger, more complex structures. However, when in the presence of non-degradable or stiff hydrogels, μ Ts lose their ability to efficiently fuse due to the constraints of the encapsulating hydrogel [66]. The main challenge of this approach is ensuring that any temporary supporting material eventually disappears, leaving behind only cell-derived tissue.

In 3D printing, two main categories of temporary and/or permanent supports are commonly employed. The first is a supporting bioink, which protects cells from shear stress during extrusion and provides initial mechanical stability after deposition [392]. The second is a sacrificial support bath, which functions as a medium that enables the printing of bioinks with lower viscosity—materials that would otherwise be difficult to extrude with precision or to maintain high-resolution structures [213]. In embedded bioprinting, a support bath is used to stabilise the deposited material during fabrication. In this work, XG-MA was employed as a rheologically suitable medium that not only enabled precise filament deposition but also provided physical boundaries that guided tissue maturation and alignment, ultimately allowing the generation of anisotropic grafts. Moreover, XG-MA has been investigated as a material that can be tailored to influence the phenotypic

response of MSC-derived μ Ts and, in doing so, direct their differentiation toward different musculoskeletal lineages [393]. Despite its advantages, XG-MA presents a critical limitation: it is not degradable under physiological conditions. Any residual XG-MA that remains trapped between printed filaments weakens the final construct and may limit its use as an implantable tissue. This creates the need for a delicate balance; the material must remain present long enough to sustain cellular maturation and tissue organisation, but it must also be removed or degraded to ensure the engineered tissue can be compacted, fused, and ultimately implanted. Addressing this challenge requires strategies that introduce bath degradability without compromising short-term structural support.

Previous studies using oxidised methacrylate alginate demonstrated that oxidation can be leveraged to tune degradation rates [356, 357]. However, even in those cases, the support bath was never fully removed during culture. Oxidising XG-MA has a similar effect, increasing its susceptibility to degradation, but the process is not well established and, in practice, does not allow the bath to degrade sufficiently to be cleared before implantation [394]. On the other hand, the use of unmodified XG as a support bath, while sacrificial [211, 319], was shown earlier in this thesis to be unsuitable on its own: its lack of structural stability leads to tissue shrinkage, loss of shape fidelity, and the inability to direct tissue maturation. To address these shortcomings, a new strategy was pursued: combining XG-MA with unmodified XG to create a semi-interpenetrating polymer network (semi-IPN). IPNs are composite materials formed when two or more polymers establish independent networks that physically intertwine without forming covalent bonds between them [395, 396]. This entanglement locks the polymers together and results in materials with mechanical and elastic properties superior to those of the individual components. For instance, IPNs of alginate and gelMA have been shown to exhibit higher elastic modulus and enhanced elasticity compared to single-network hydrogels [397-399]. In this approach, mixtures of XG-MA and XG were crosslinked under UV light in the presence of LAP, resulting in the unmodified xanthan chains becoming physically trapped within the crosslinked bath. This design couples the structural stability of the methacrylate network with the sacrificial properties of native xanthan, potentially generating a support bath with tuneable degradability that could be suited for embedded printing applications.

Building on this rationale, this work aimed to adapt XG-MA in a way that preserved its favourable physical properties while simultaneously introducing a controlled degree of degradation. To achieve this, we formulated mixtures of XG-MA and unmodified XG at different ratios and conducted an initial screening to evaluate their rheological characteristics and their performance in μ T-based embedded printing. Based on these results, four formulations were selected for biological assessment, focusing on their ability to support fibrochondrogenesis and collagen fibre alignment to ensure that tissue anisotropy was preserved even as the bath degraded. Once the optimal formulation was identified, the system was scaled up to fabricate $\sim 1 \times 1$ cm anisotropic sheets for meniscal TE applications. These constructs were then subjected to biaxial mechanical testing to determine whether they exhibited the structural integrity and mechanical functionality required for potential translation into preclinical studies.

6.2 Materials and Methods

6.2.1 Isolation and expansion of gBM-MSCs and μ T formation

Bone marrow-derived MSCs were isolated from the sternum of a skeletally mature female goat. The extracted marrow was washed in expansion medium containing high-glucose Dulbecco's Modified Eagle Medium (hgDMEM), 10% foetal bovine serum (FBS) and penicillin (100 U/mL) – streptomycin (100 μ g/ml) (all from Gibco) and triturated with a 16G needle until a homogenous mixture was obtained. The suspension was then centrifuged at 650 g for 5 min, and the resultant cell pellet was resuspended in fresh expansion medium twice before it was filtered through a 40 μ m cell sieve (Sarstedt). Cell counting was performed with trypan blue in the presence of acetic acid (6% final) before plating at a density of 1.3×10^5 cells/cm². Following colony formation, cells were trypsinized, counted, and replated for 2 additional passages at a density of 5×10^3 cells/cm² at 5% pO₂ in expansion medium supplemented with 5 ng/ml of fibroblast growth factor (FGF)- 2 (PeproTech Ltd). Medium change was performed three times per week. The μ Ts were fabricated as previously described (Chapter III). The 3D printed mould stamps containing 1,889 micro resections and 4% (w/v) ultrapure agarose were sterilised in an autoclave at 120°C for 20 minutes. Next, 6 mL of ultrapure agarose was gently poured into each well of

the 6-well plate and allowed to cool down for 15 min after moulding it. Agarose moulds were then soaked overnight in XPAN, at 37°C in a humidified atmosphere with 5 % CO₂ and 5 % O₂. MSCs were seeded at a density of $7,55 \times 10^5$ into the microwells in a final volume of cell suspension of 5 mL of CDM+. 20 minutes after seeding, the plates were centrifuged for 5 minutes at 700 x g to collect cells at the bottom of each well. After 2 days, μ Ts were harvested as described before [134], and used at a density of 45,000 μ Ts/mL for bioprinting experiments.

6.2.2 Synthesis of support bath formulations

Xanthan gum (XG; Sigma, from *Xanthomonas campestris*) was dissolved at the prescribed concentration (w/v) in phenol red-free DMEM (Gibco) and rotated overnight at room temperature until fully dissolved. XG-MA was prepared as previously described[232]. Briefly, XG was dissolved at 0.5% (w/v) in ultrapure water with stirring until fully solubilised, then glycidyl methacrylate (40 mL) was added and the mixture was stirred overnight at 60 °C, protected from light. The product was dialysed against deionised water for 7 days with twice-daily water changes, lyophilised for 48 h, and stored at -20 °C. For sterilisation, weighed aliquots were exposed to ethylene oxide gas (Andersen Products) for 12 h. Two days before printing, XG-MA was dissolved in phenol red-free DMEM supplemented with penicillin (100 U/mL) and streptomycin (100 μ g/mL), and rotated at 40 rpm at room temperature for 48 h. On the printing day, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP; Sigma) was added to a final concentration of 0.25% (w/v) and vortexed to mix. For mixed formulations, fully dissolved components were combined at the prescribed ratios and vortexed until homogeneous. To remove entrapped air, baths were centrifuged at $2,500 \times g$ for 5 min at room temperature. For each print, 4 mL of XG-MA was dispensed into a 6-well plate using a positive-displacement pipette (Microman E, Gilson) to minimise bubble formation.

6.2.3 Degradation and swelling tests of support baths

For degradation and swelling studies, gels were prepared using custom-built moulds. A negative PLA mould was built using an SLS printer (Prusa). For the positive mould, PDMS was poured into the mould and cured at 80 °C inside an oven. The XG, XG-MA and XG: XG-MA (1ml per gel) were pipetted inside each well of the positive mould, and crosslinked

under UV light (405 nm) for 4 min. Therefore, the gels were dissolved in phenol-free DMEM supplemented with 1% pen strep and the media was changed twice a week. All in sterile conditions. At each time point, wet weight was recorded, and the gels were freeze-dried for 48 hours, and the dry weight was recorded.

6.2.4 Rheological measurements of XG: XG-MA bath formulations

Rheological characterisations were conducted on an MCR 102 Rheometer (Anton-Paar, Hertford Herts, UK) 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 as previously described (Chapter IV). The viscosity as a function of shear rate ($0.1\text{--}1000\text{ s}^{-1}$) was measured at RT for the support bath formulations. The support baths were maintained under high-humidity conditions to minimise dehydration during testing, and all rheological measurements were carried out in triplicate.

6.2.5 Bioprinting of μ Ts into XG: XGMA support baths

For bioprinting experiment, μ Ts were harvested from agarose microwells after 2 days, transferred to a 50 mL tube with culture medium, and centrifuged at $650 \times g$ for 5 min at room temperature to pellet the μ Ts. Gelatin 1% (w/v) was then added to achieve a final density of 45,000 μ Ts/mL. The bioink was loaded into sterile printing cartridges and kept at 4 °C for 15 min to ensure homogeneous gelation. Printing was performed under aseptic conditions using a syringe-pump printhead (CELLINK, Sweden) at an extrusion rate of $6\text{ }\mu\text{L s}^{-1}$ and a print speed of 2 mm s^{-1} . Two geometries were fabricated: a single 10 mm filament, and a “tissue sheet” comprising 15 parallel 10 mm filaments within a $10 \times 10\text{ mm}$ area, spaced 600 μm centre-to-centre to prevent filament fusion. G-code was generated in DNA Studio 4 (CELLINK).

6.2.6 Live/Dead analysis

Cell viability was assessed using a live/dead assay kit (Invitrogen) as per manufacturer's instructions. Briefly, constructs were rinsed with PBS, incubated for 1 hour at 37 °C in a staining solution containing calcein (2 μM , excitation 485 nm, emission 530 nm) and Ethidium Homodimer (4 μM , excitation 530 nm, emission 645 nm), and then rinsed again in PBS to remove excess dye. Samples were imaged with a Leica SP8 confocal microscope,

and maximum projection z-stacks were generated to evaluate viability throughout the tissue depth.

6.2.7 Histological Evaluation

For construct analysis, constructs were retrieved by mechanically disrupting the surrounding support bath and carefully extracting the samples using tweezers. Samples were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. After fixation, they were dehydrated through a graded ethanol series (50–100% v/v), cleared in xylene, and embedded in paraffin wax (Sigma) as previously described [11]. Sections of 5 µm thickness were cut, rehydrated, and subjected to histological staining. Haematoxylin and eosin (H&E; Sigma) was used to assess tissue morphology, ECM, and nuclei. Sulphated glycosaminoglycan (sGAG) content was stained with 1% (w/v) Alcian Blue (AB) 8GX in 0.1 M HCl and counterstained with 0.1% (w/v) nuclear fast red for cell distribution. Collagen deposition was visualised using 0.1% (w/v) Picrosirius Red (PR; Sigma). Stained sections were mounted in Pertex (Avantor) and imaged with an Aperio ScanScope slide scanner (Leica, Germany).

6.2.8 Biochemical evaluation

Samples were washed twice in 1× PBS, dried, and digested at 60 °C for 18 h in papain (3.88 U/mL) prepared in 100 mM sodium phosphate, 5 mM Na₂EDTA, and 10 mM L-cysteine, pH 6.5 (all Sigma). DNA was quantified with the Quant-iT PicoGreen dsDNA kit (Molecular Probes, Biosciences). Sulphated GAGs were measured by DMMB assay (dye pH 1.5) using a 1 mg/mL chondroitin sulphate standard; absorbance at 530 and 590 nm was recorded on a Synergy HT microplate reader (BioTek), and the 530/590 ratio was used for the standard curve and sample calculations. Total collagen was determined via hydroxyproline using a chloramine-T/DMAAB assay: samples were mixed 1:1 with 38% HCl and hydrolysed at 110 °C for 18 h, cooled, centrifuged (5,000 × g, 5 min), dried at 60 °C for 48 h, and reconstituted in 200 µL ultrapure water. Chloramine-T (2.82% w/v) and 4-(dimethylamino)benzaldehyde (0.05% w/v) were added and incubated for 20 min at room temperature in the dark, then read at 570 nm against a trans-4-hydroxy-L-proline standard curve. Collagen content was calculated using a hydroxyproline-to-collagen mass ratio of 1:7.69.

6.2.9 Immunofluorescence evaluation

Following fixation in 4% paraformaldehyde overnight and paraffin processing (Section 6.2.5), sections were rehydrated through graded ethanol (100–50%) into 1× PBS (Gibco). Antigen retrieval was performed enzymatically with Hyaluronidase (4,000 U/mL, 25 min, 37 °C; Sigma-Aldrich) followed by Pronase (3.5 U/mL, 25 min, 37 °C, Sigma). Sections were rinsed and blocked for 1 h at room temperature in PBS containing 1% BSA and 10% goat serum. Primary antibodies to collagen I (Abcam, ab90395; 1:400) and collagen II (Santa Cruz, sc-52658; 1:400) were applied in blocking buffer and incubated overnight at 4 °C in a humidified chamber. After three PBS washes, appropriate secondary antibodies in 2% BSA were applied for 1 h at room temperature in the dark. Slides were washed, mounted with Fluoroshield containing DAPI (Sigma), sealed, and stored at 4 °C in the dark. Imaging was performed on a Leica SP8 confocal microscope; representative images are shown as maximum-intensity projections of z-stacks.

6.2.10 Biaxial tensile testing of printed tissues

Stress-relaxation tensile tests were performed on a CellScale biaxial testing system (operated in uniaxial mode) with a 10 N load cell. Square specimens (10 × 10 mm) were printed specifically for tensile testing. Samples were mounted and submerged in 1× PBS throughout. A preload of 0.02 N was applied, followed by a displacement-controlled ramp at 0.5 mm·min⁻¹ to 8% nominal strain, then a 10 min hold to reach quasi-equilibrium. Ramp and equilibrium moduli were calculated as the slopes of the engineering stress–strain curve in the linear regions immediately before the hold and near the end of the hold, respectively.

Surface strains were quantified by DIC during stress-relaxation tensile testing. Before mounting, the specimen surface was gently blotted and coated with a high-contrast random speckle pattern using graphite. Images were captured with a monochrome camera aligned normal to the specimen surface. Engineering stress was calculated as force divided by the initial cross-sectional area. Ramp and quasi-equilibrium moduli were obtained by linear regression of the engineering stress–strain response in the linear regions immediately before the hold and near the end of the hold, respectively, using DIC-derived axial strain to minimise machine-compliance artefacts. All tests were performed in triplicate.

6.2.11 Image quantification analysis of immunofluorescence pictures and PLM analysis

For immunofluorescence quantification, the intensity of 3 specific regions of interest (ROI) within each sample was measured and quantified through ImageJ software. The intensity was then normalised by the number of nuclei in the ROI. For PLM analysis, Sections were stained with 0.1% (w/v) Picrosirius Red, mounted with DPX (Sigma-Aldrich), and imaged on an Olympus BX41 polarising light microscope equipped with a MicroPublisher 6 CCD camera and an Olympus U-CMAD3 adapter. Collagen fibril orientation and coherency were quantified in ImageJ using the OrientationJ and Directionality plugins.

6.2.12 Statistical analysis

Analyses were performed in GraphPad Prism (GraphPad Software, San Diego, CA, USA). Group differences across time were tested with two-way ANOVA followed by Tukey's post hoc multiple comparisons. Differences between groups at a single time point were tested with one-way ANOVA followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$. Significance was accepted when $p < 0.05$. Graphical results were designed with GraphPad Prism.

6.3 Results

6.3.1 XG: XG-MA baths support high-fidelity printing with controlled bath degradation

To evaluate the printability of this new, potentially more degradable support bath formulation, we tested different ratios of unmodified XG and XG-MA. The rheological properties of hydrogels with relatively low (0.14:1, XG: XG-MA) and high (7:1, XG: XG-MA)

ratios of XG: XG-MA were first compared to assess their suitability as support baths for embedded bioprinting (Fig. 6.1).

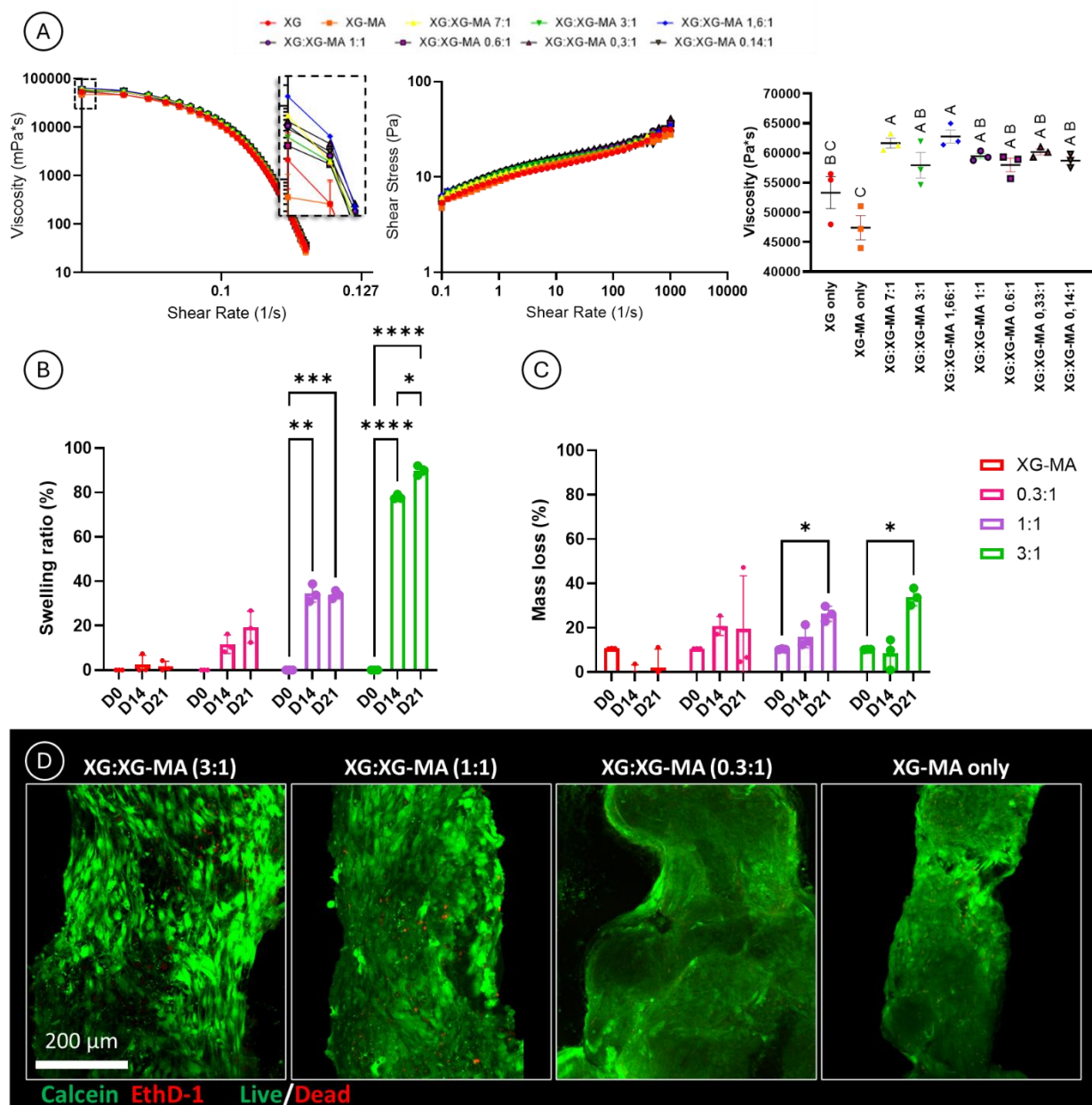


Figure 6.1. Rheological properties and cell viability of XG and XG-MA hydrogels.

(A) Viscosity of XG, XG-MA, and XG: XG-MA blends across shear rates of 0.1–10,000 s⁻¹, and zero-shear viscosity of the corresponding support baths. Group differences were assessed by one-way ANOVA with Tukey's multiple comparisons test; no significant differences were detected. (B) Swelling ratio (%) and (C) mass loss (%) for the selected blend ratios and XG-MA at day 0 (D0), day 14 (D14), and day 21 (D21). Swelling ratio was calculated as: $(W \text{ (mg) wet} - W \text{ (mg) dry}) / (W \text{ (mg) dry}) \times 100$ at each time point. Mass loss was calculated as: $((W \text{ (mg) Day 0} - W \text{ (mg) Day X}) / W \text{ (mg) Day 0}) \times 100$, where W Day 0 is the initial dry weight, and W Day X is the dry weight at the given point. Differences across groups and time were evaluated by two-

way ANOVA with Tukey's post hoc test. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (D) Live/dead staining of printed μ Ts in the selected XG: XG-MA blends at day 28. Scale bar: 200 μ m.

All formulations exhibited similar favourable shear-thinning profiles (Fig. 6.1 A). Closer inspection revealed minor differences in viscosity at low shear stress; however, when comparing absolute viscosity at low shear rates, no significant differences were observed among the groups (Fig. 6.1 A). Therefore, blending unmodified XG into XGMA does not substantially alter the rheological behaviour compared to XGMA alone, suggesting such blends provide similar short-term mechanical and rheological characteristics as the previously used XGMA support bath—while offering a potentially more sacrificial nature. After characterising the rheological properties across the full range of formulations, cell viability after bioprinting into three different baths was assessed: one with a low XG content (0.3:1), one intermediate (1:1), and one high (3:1). All three baths supported high cell viability at day 28, with no clear differences between the blend and the XG-MA baths. To further assess the sacrificial character of the materials, we conducted degradation and swelling studies by maintaining the selected blends under humidified, physiological conditions for 21 days (37 °C). The incorporation of unmodified XG influenced the swelling behaviour during the first 14 days, showing a clear correlation between increasing XG content and greater swelling (Fig. 6.1 B). Higher swelling reflects increased water uptake and network relaxation, indicative of a lower crosslink density that facilitates degradation. This relationship between swelling and mass loss aligns with degradation mechanisms where solvent penetration precedes polymer chain cleavage. In our study, materials with higher XG generally exhibited increased swelling over time and a tendency toward faster degradation compared to formulations with lower XG content (Fig. 6.1C), although this relationship was not strictly consistent across all conditions. This behaviour likely reflects

the interplay between reduced crosslink density and physical entanglements within the network, which can transiently stabilise intermediate formulations (Fig. 6.1 C).

6.3.2 XG: XG-MA support chondrogenesis over multiple ratios for meniscus TE

The capacity of the different support baths to support fibrochondrogenesis post-printing was next assessed. To this end, microtissues printed into 3:1, 1:1 and 0.3:1 XG: XGMA support baths were maintained in culture for 28 days in CDM+ (Fig. 6.2).

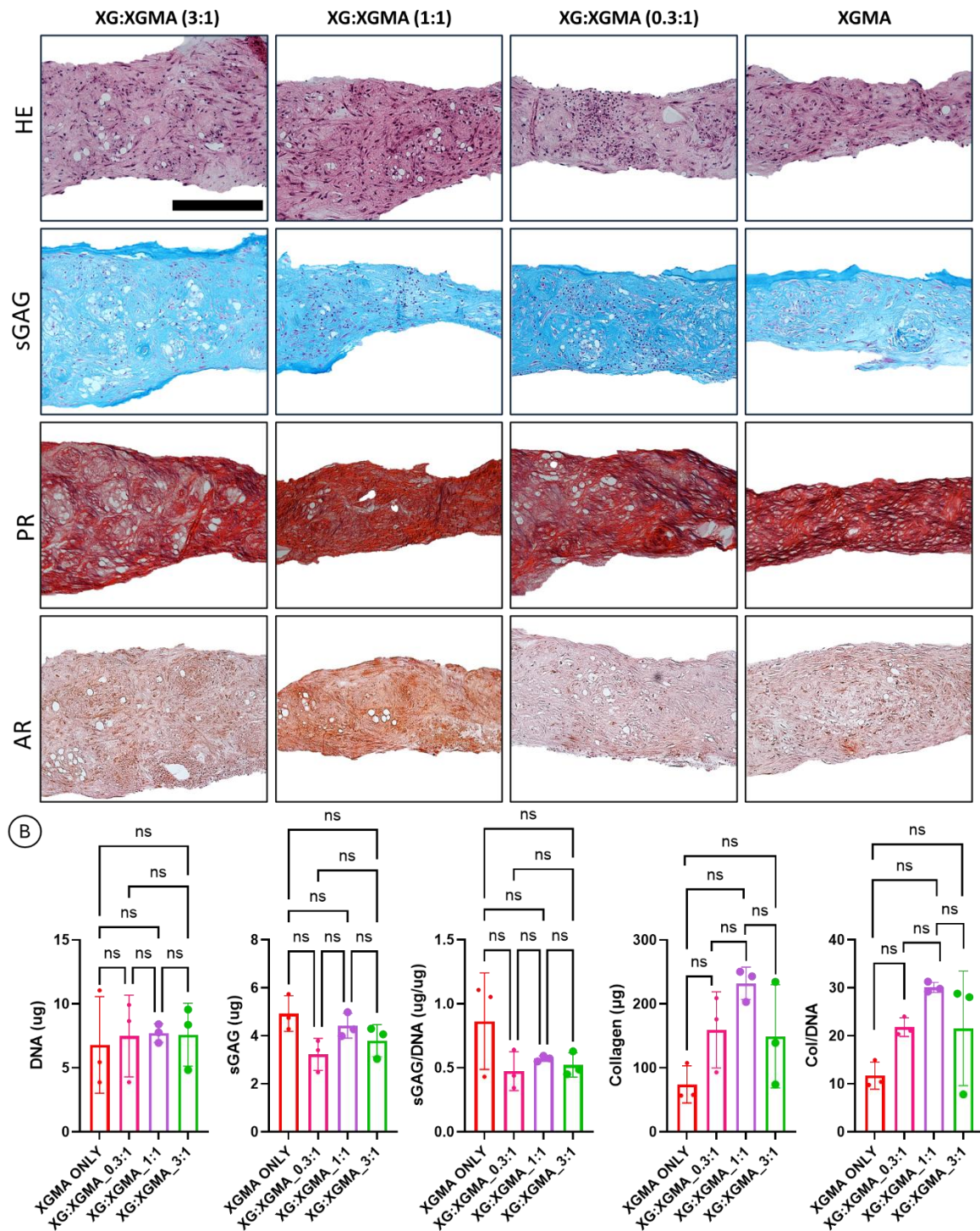


Figure. 6.2. XG: XG-MA supports the chondrogenic phenotype of biprinted μ Ts at day 28 of culture in chondrogenic media. A) histological staining for H&E, sGAG (AB), Collagen (PR) and Calcium deposit (AR) at day 28 of biprinted μ Ts showing high sGAG and collagen deposition and negativity for calcium staining. B) biochemical analysis of biprinted μ Ts at day 28 showing no significant differences between groups. Group differences were assessed by one-way ANOVA with Tukey's multiple comparisons test; no significant differences were detected.

Histological staining revealed that all bath formulations, regardless of the XG: XG-MA ratio, supported the development of a fibrocartilage-like tissue that was rich in sGAGs and collagen (Fig. 6.2 A). Limited calcium staining was observed across most groups, although some localised mineral deposition was present in the XG:XG-MA (1:1) formulation, likely due to specific culture conditions (Fig. 6.2 A). This consistency across conditions indicates that variations in the ratio of unmodified XG to XG-MA do not significantly influence cell phenotype or differentiation outcome. These findings were further confirmed by biochemical analyses, which demonstrated comparable levels of DNA content, sGAG accumulation, and collagen production across all formulations (Fig. 6.2 B). Therefore, while modifying the relative proportion of XG and XG-MA can tune the material's degradability and swelling characteristics, such compositional changes do not compromise the biological

performance of the support bath or its capacity to support (fibro)chondrogenic differentiation of the embedded μ Ts.

6.3.3 Increasing the XG fraction in XG: XG-MA support baths reduces collagen fibre alignment following μ T bioprinting

To further examine the structural organisation of the newly formed tissue, PLM was performed on printed filaments after 28 days of culture to quantify collagen fibre orientation and coherency across the different XG: XG-MA ratios (Fig. 6.3).

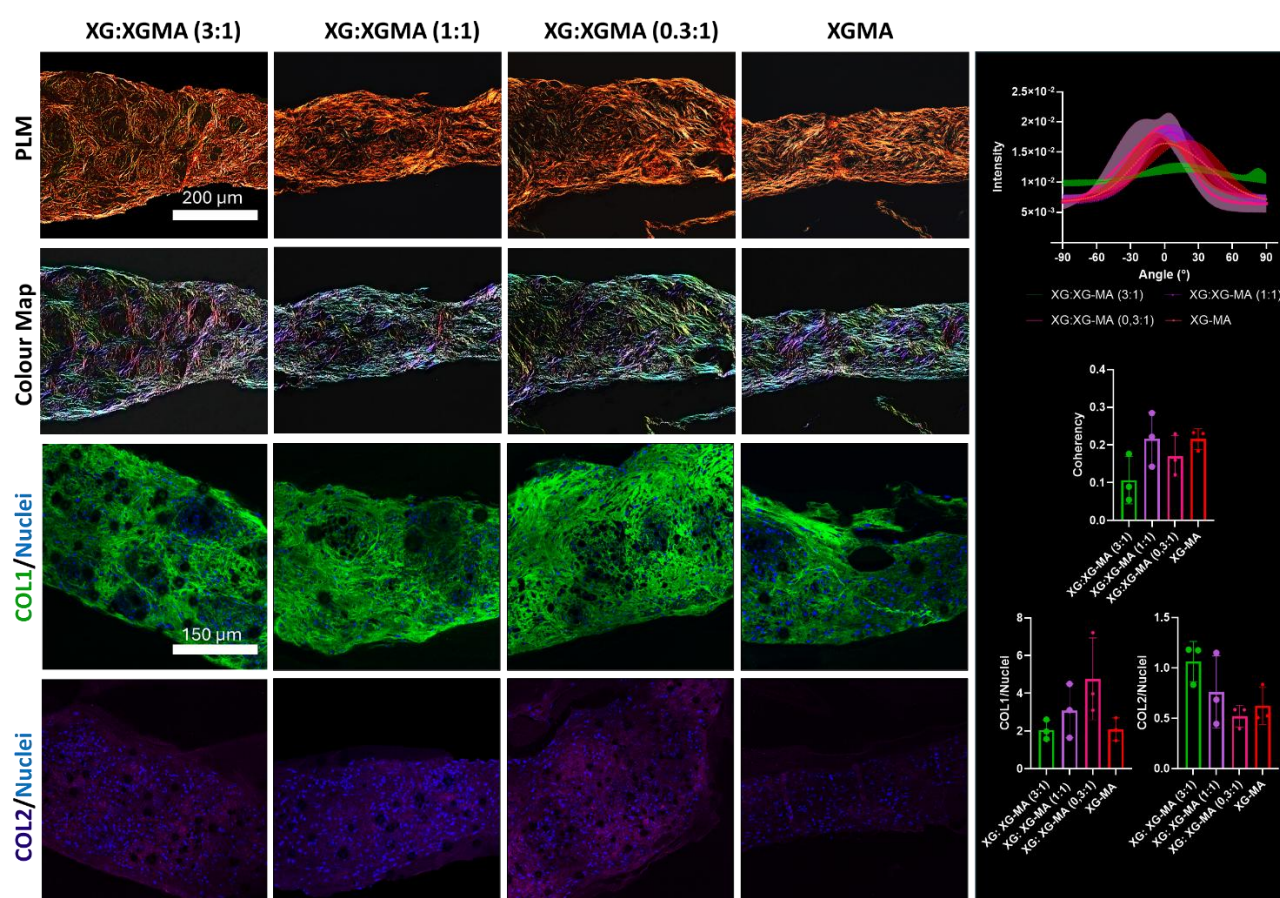


Figure 6.3. Higher XG content in the support-bath formulation diminishes collagen alignment into a single preferred direction, reducing tissue anisotropy. (A) Polarised light microscopy (PLM) of printed μ Ts at day 28 after Picrosirius Red staining, with corresponding fibre-orientation colourmaps. Colour hue encodes orientation: blue/cyan $\approx 0^\circ$ and pink/red $\approx 90^\circ$. Scale bar: 200 μ m. Immunofluorescence for specific collagen deposition at day 28, counterstained with DAPI. Scale bar: 150 μ m (B) Quantification of fibre orientation for each support bath. (C) Fibre coherency, where values near 1 indicate strong alignment and values near 0 indicate greater dispersion ($n = 3$). (D–E) Quantification of immunofluorescence signal for the support baths, normalised to the number of nuclei per region of interest (ROI) ($n = 3$). Group differences in panels C–E were

assessed by one-way ANOVA with Tukey's multiple comparisons test; no significant differences were detected.

PLM analysis revealed that increasing the proportion of unmodified XG altered tissue anisotropy post-printing (Fig. 6.3 A). A marked reduction in birefringence intensity was observed in the 3:1 (XG: XG-MA) group compared to the other formulations, indicating lower alignment of collagen fibres (Fig. 6.3 B). Quantitative assessment confirmed a decrease in fibre coherency in the 3:1 group relative to all other conditions (Fig. 6.3 C). Abundant type I collagen deposition was observed across all groups, with slight variations in type II collagen deposition observed, although these were not statistically significant (Fig. 6.3 A, D, E). Based on these findings, we selected the formulation that displayed a more sacrificial behaviour without compromising the anisotropy of the tissue. The 1:1 (XG: XG-MA) ratio fulfilled these criteria, maintaining appropriate rheological and structural properties while supporting consistent chondrogenic differentiation, and was therefore used for all subsequent experiments.

6.3.4 Optimised XG: XG-MA support bath enhances inter-filament fusion, although complete construct integration remains limited

After identifying the optimal support bath formulation, we next focused on scaling up the construct to design and bioprint anisotropic tissues. To achieve this, filaments were printed in parallel and spaced closely enough to allow subsequent fusion, while maintaining sufficient separation to preserve distinct filament alignment and overall anisotropy. A series of filaments was printed to form a 1 cm × 1 cm square sheet (Fig. 6.4).

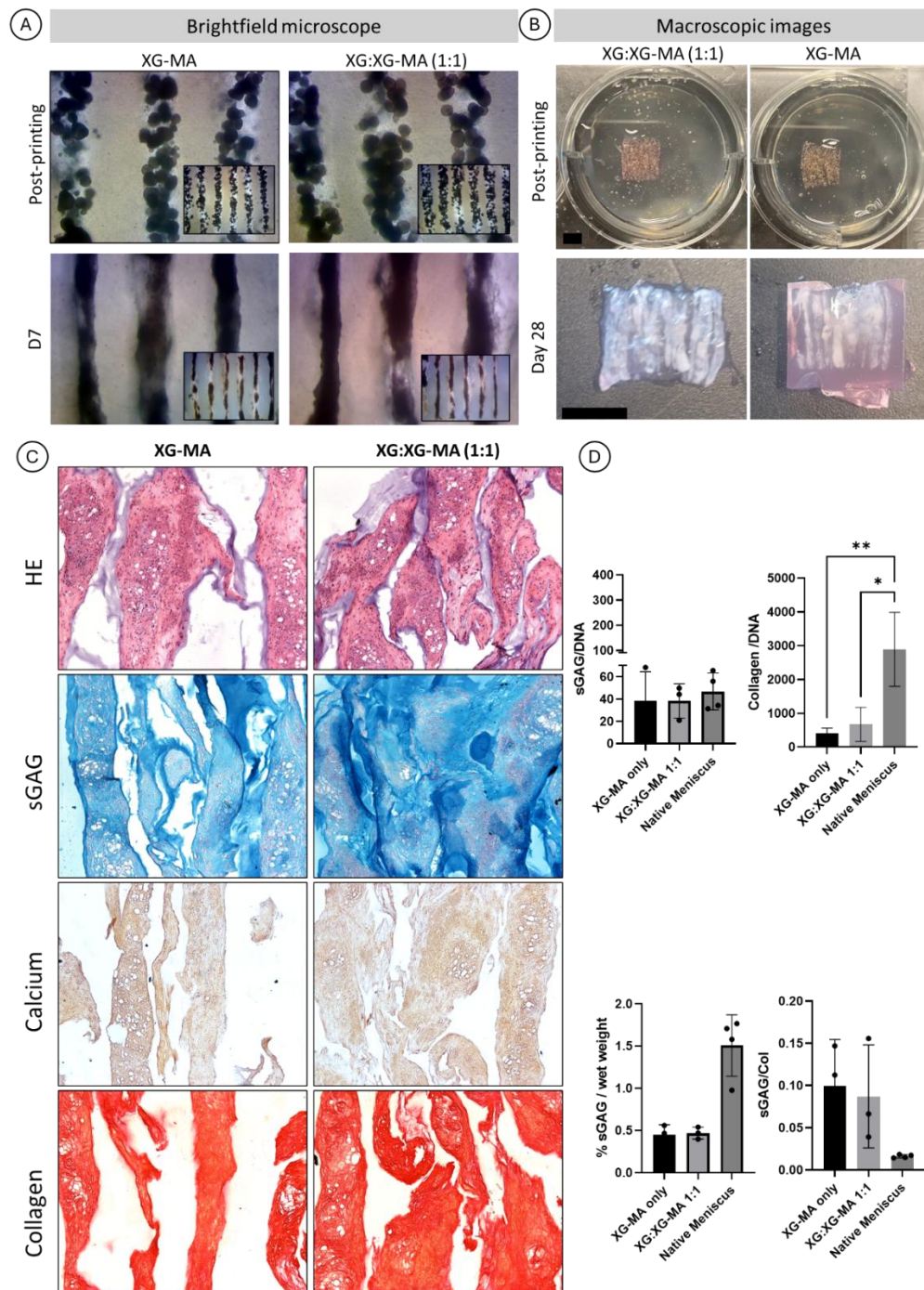


Figure 6.4. Incorporating XG at a 1:1 XG: XG-MA ratio promotes support-bath degradation and enhances inter-filament fusion in scaled-up tissue sheets. (A) Brightfield micrographs of printed tissue sheets immediately post-printing (D0) and at day 7 (D7), showing progressive fusion of printed μ Ts. (B) Macroscopic photographs of tissue sheets at D0 and day 28 (D28); the 1:1 XG: XG-MA blend exhibits improved fusion between filaments, yielding a more cohesive graft. Scale bar: 0.5 cm. (C) Histology at D28: H&E, sulphated GAGs (Alcian Blue, AB), calcium deposits (Alizarin Red, AR), and total collagen (Picrosirius Red, PR). (D) Biochemical analysis at D28 versus native meniscus: sGAG/DNA, collagen/DNA, %sGAG per wet weight, and

sGAG/collagen. Group differences were evaluated by one-way ANOVA with Tukey's multiple comparisons test; significance was accepted at $p < 0.05$. * $p < 0.05$, ** $p < 0.01$.

The rationale behind this design was that, following printing, individual μ Ts would first undergo fusion within each printed filament, establishing local anisotropy. As the support bath gradually degraded, the neighbouring filaments would then move closer and eventually fuse, resulting in a continuous yet directionally organised tissue. This approach was intended to balance two key processes: the early fusion of μ Ts within each filament to initiate anisotropic organisation, and the later fusion of adjacent filaments to generate a compact, macroscopically aligned construct while preserving overall directional architecture. Fusion of μ Ts within each filament was already visible after 7 days of culture (Fig. 6.4 A). Over 28 (Fig. 6.4 B) and 42 days (Fig. 6.4 C), distinct differences emerged between the support bath formulations. In the XG-MA bath, the printed filaments maintained their original spacing, and no substantial structural changes were observed throughout culture (Fig. 6.4 B). In contrast, constructs printed in the XG: XG-MA blend exhibited progressive fusion of adjacent filaments as the bath degraded, leading to a denser and more compact tissue structure while maintaining the original printing path (Fig. 6.4 B). Chondrogenic staining of the scaled-up constructs revealed no qualitative differences between the two support bath formulations, consistent with the results obtained for single printed filaments, confirming that chondrogenesis was maintained across different experimental conditions and scales (Fig. 6.4 C). Examination of the overall tissue morphology highlighted a clear distinction in filament spacing between the two groups. In the XG-MA-only constructs, the filaments remained separated, preserving their original print path and spacing, whereas in the XG: XG-MA blend, the filaments appeared more fused, forming a denser and more cohesive tissue (Fig. 6.4 C). The filaments in the blended group also appeared larger in cross-section, suggesting later-stage inter-filament fusion that contributed to a more compact and cohesive construct. Biochemical analysis showed no significant differences between the two groups in terms of DNA, sGAG, or total collagen content (Fig. 6.4 D). However, the collagen-to-DNA ratio differed from that of native meniscal tissue (Fig. 6.4 D).

To determine whether the increased filament fusion observed in the blended group affected tissue anisotropy, collagen fibre orientation was analysed by PLM (Fig. 6.5).

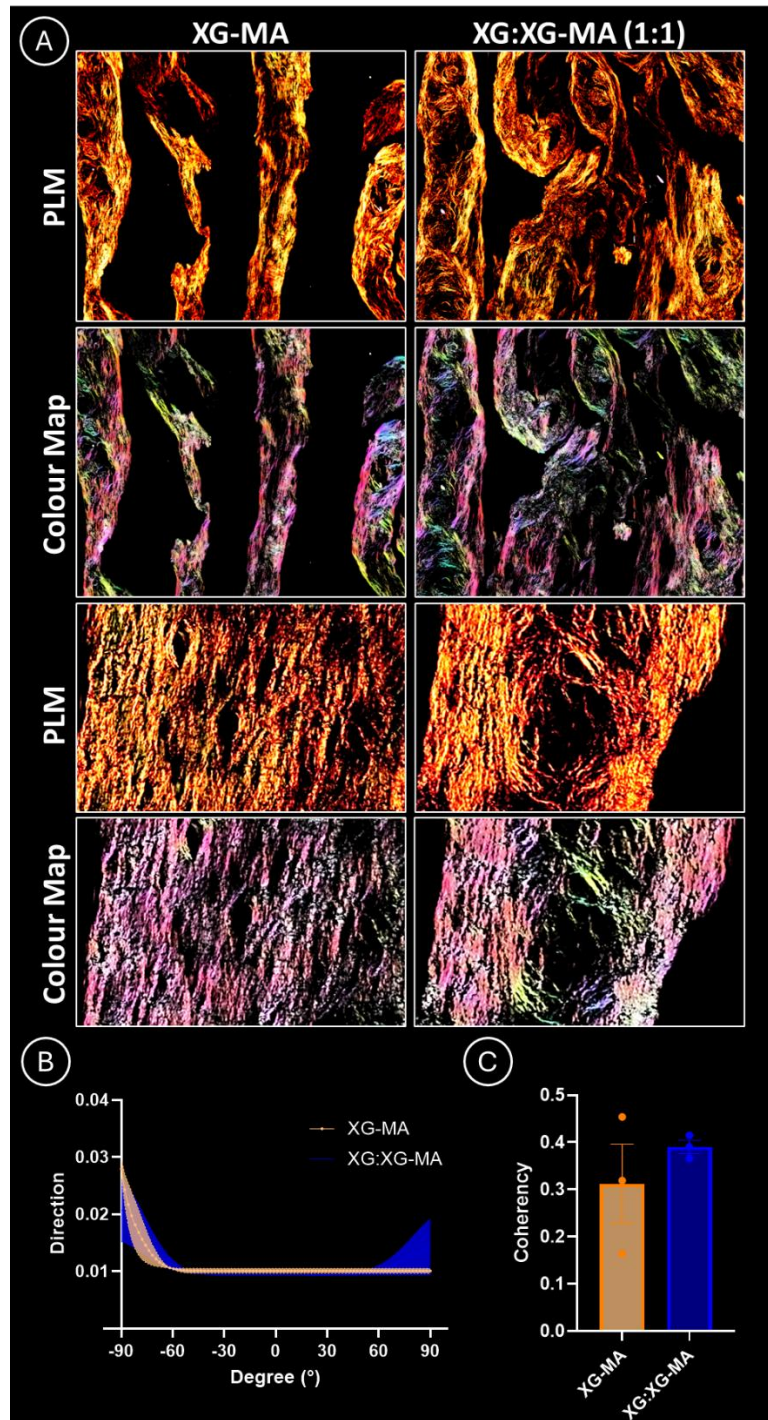


Figure 6.5. XG: XG-MA support baths promote inter-filament fusion while preserving anisotropy in scaled-up tissue sheets at day 28. (A) Polarised light microscopy of Picrosirius Red–stained tissue sheets at day 28 with corresponding fibre-orientation colour maps; hue encodes orientation (blue/cyan $\approx 0^\circ$, pink/red $\approx 90^\circ$). Insets

show higher-magnification views of individual filaments, highlighting strong intra-filament anisotropy with matching colour maps. (B) Quantification of fibre-orientation distributions for each support bath. (C) Fibre coherency, where values approaching 1 indicate strong alignment and values near 0 indicate greater dispersion (n = 3).

In the XG-MA-only group, individual filaments were clearly distinguishable, exhibiting a well-defined anisotropic pattern with collagen fibres predominantly aligned around 90° and −90°, as shown by distinct orientation peaks (Fig. 6.5 A, B). The XG: XG-MA blended constructs displayed a similar alignment pattern, with collagen fibres primarily oriented along the same directions, indicating preservation of the global anisotropic organisation despite filament fusion (Fig. 6.5 A, B). Coherency analysis further confirmed that there were no significant differences between the two formulations (Fig. 6.5 C). Closer inspection of individual filaments showed highly coherent fibre alignment in both groups, demonstrating that the collagen network maintained its structural organisation independent of bath composition (Fig. 6.5 A, bottom panels). These results confirm that scaled-up grafts can be successfully engineered using both bath formulations and that accelerating the degradation of the support bath does not compromise either chondrogenic differentiation or anisotropy of the final construct.

6.3.5 Tuning the XG: XG-MA support bath increases inter-filament cohesion and tensile strength in printed meniscal grafts

To further characterise the mechanical properties and confirm the anisotropic behaviour of the engineered constructs, biaxial tensile testing was performed (Fig. 6.6).

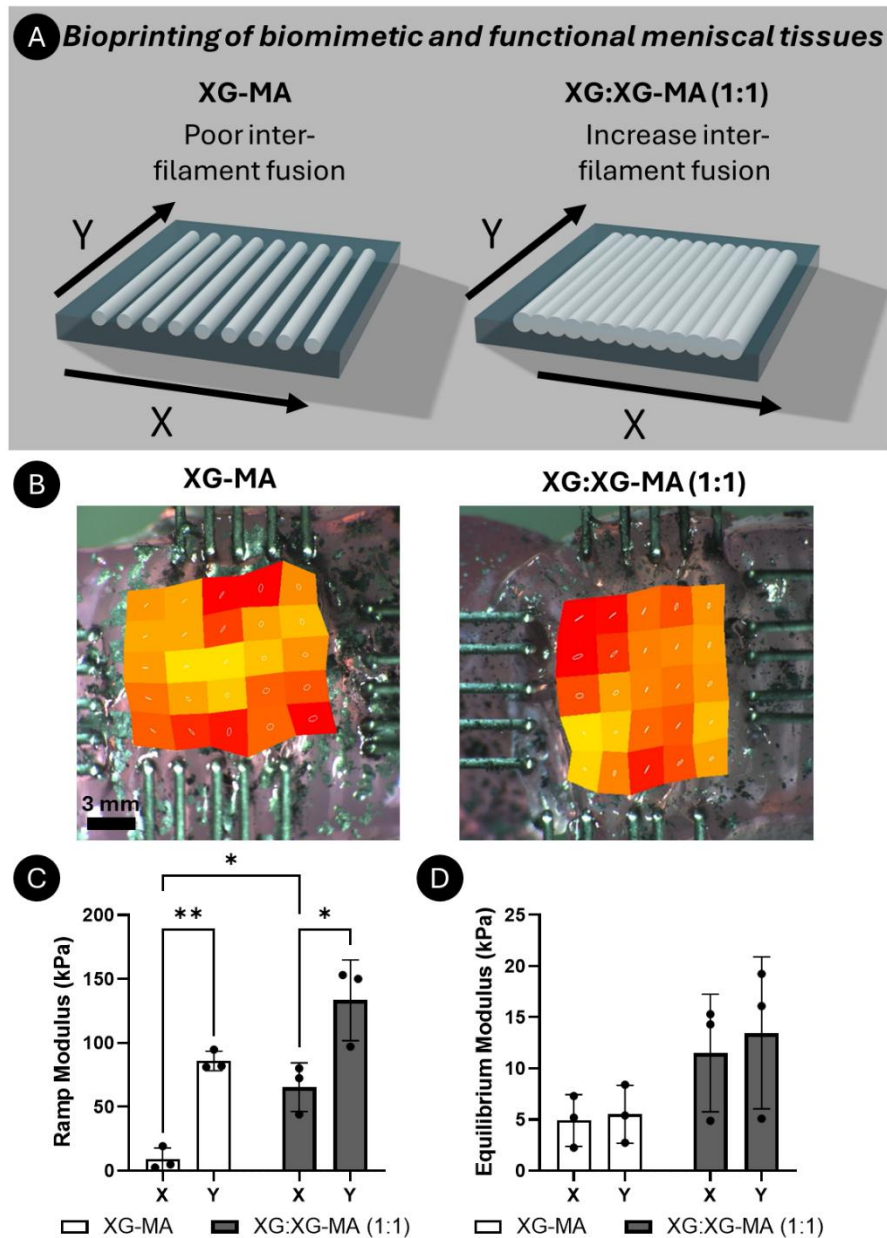


Figure 6.6. Degradability of the XG:XG-MA support bath enables the fabrication of mechanically competent bioprinted meniscal tissues. (A) Schematic of the bioprinting design and resulting constructs: poor inter-filament fusion yields tissues that remain anisotropic along the y-axis but are mechanically weak and poorly integrated along the x-axis, whereas improved inter-filament fusion preserves anisotropy while increasing cohesion and Young's modulus along the x-axis. (B) Macroscopic images of the tensile testing setup with a displacement grid highlighting regions of highest (red) and lower (yellow) deformation. Scale bar: 3 mm. (C) Ramp modulus of printed tissue sheets in the x- and y-directions. (D) Equilibrium modulus of printed tissue sheets in the x- and y-directions. Statistical significance was assessed by two-way ANOVA followed by a post hoc multiple comparison test. Significance was accepted at $p < 0.05$. * $p < 0.05$; ** $p < 0.01$.

This approach was selected because the meniscus experiences multidirectional loading *in vivo*, and biaxial testing allows assessment of mechanical performance along both the primary (longitudinal or Y direction) and secondary (transverse or X direction) axes of the printed tissue. The test also served as a proof of concept to evaluate the degree of filament fusion, with greater fusion between filaments manifesting as a higher stiffness in the X direction. The mechanical data demonstrated clear anisotropy in all constructs, with higher Ramp and Equilibrium Moduli along the y-axis (Fig. 6.6 C, D), corresponding to the longitudinal orientation of the printed filaments, and lower moduli along the perpendicular x-axis (Fig. 6.6 C, D). Comparison between the two formulations (XG-MA and XG: XG-MA) revealed that constructs printed in the XG: XG-MA blend exhibited increased stiffness along the short axis (≈ 50 kPa) relative to the XG-MA-only group (≈ 10 kPa), indicating greater inter-filament fusion and improved load transfer between adjacent filaments (Fig. 6.6 C, D). These results confirm that the printed tissues retain anisotropic mechanical behaviour consistent with their designed architecture, while the partially sacrificial XG: XG-MA blend promotes enhanced structural integration without loss of directional organisation.

6.4 Discussion

This study demonstrates that blending XG-MA with unmodified XG to form a semi-IPN provides a controllable strategy to modulate degradability in embedded bioprinting support baths, without compromising rheological properties, print fidelity, or chondrogenic outcomes. The rationale behind this approach was to expand the functional “window of trade-off” between structural stability and degradability, ensuring that the support bath could both sustain printing precision and be gradually removed to allow tissue fusion and maturation.

Support baths should possess rheological properties compatible with embedded bioprinting, and where necessary, provide structural support during *in vitro* culture. Previous studies have explored various support bath systems, including granular hydrogels, which can present limitations for long-term applications, such as thermal sensitivity [314], rapid and uncontrolled degradation [211], and poor transparency [204]. In light of these constraints, a new material blend based on XG and XG-MA was investigated to provide both

structural stability and a slower degradation *in vitro*. Rheological analysis confirmed that incorporating unmodified XG across a wide range of ratios did not significantly alter the shear-thinning behaviour or viscosity profile of the resulting bath. This finding is crucial, as it ensures that the modified bath maintains the same extrusion stability and support capacity required for μ T-based embedded printing [393]. Comparable rheological properties across formulations indicate that the materials behaved similarly during extrusion. However, this does not exclude the influence of other physicochemical parameters on subsequent tissue compaction. Variations in hydrogel mesh size, crosslinking density, and network structure are likely to influence both the diffusion of soluble factors and the retention of newly deposited ECM components [400, 401]. A denser network (higher XG-MA content) may restrict the transport of nutrients, signalling molecules, and ECM macromolecules [402], while also limiting ECM remodelling, thereby limiting inter-filament fusion. Conversely, increasing the proportion of unmodified XG is expected to reduce effective crosslink density and increase mesh size, facilitating solvent uptake, enhancing mass transport and enabling greater ECM remodelling and inter-filament fusion. The degradation and swelling data established a clear relationship between increasing XG content and enhanced hydrogel relaxation, consistent with reduced crosslink density in the semi-IPN. The observed increase in swelling with higher proportions of unmodified XG relative to XG-MA suggests that the incorporation of less crosslinked, more hydrophilic XG promotes enhanced water uptake and loosening of the network structure. In addition, the highly anionic nature of XG-based systems [403] may contribute to electrostatic interactions with charged ECM components [403], such as GAGs and collagen, potentially influencing their spatial distribution and retention within the construct. These interactions, together with different degradation kinetics, may further modulate the local microenvironment experienced by the fusing μ Ts. However, this trend did not scale linearly across all formulations, likely reflecting the complex interplay between physical entanglements and chemical crosslinks within the system. At intermediate ratios, interactions between XG and XG-MA may transiently stabilise the network, resulting in a non-linear swelling and degradation behaviour. Therefore, the observed behaviour likely reflects a combination of degradation dynamics, evolving mesh structure, and physicochemical interactions, rather than degradation alone.

Previous studies have investigated the degradation properties of an oxidised methacrylate alginate (OMA) support bath and its effect on chondrogenesis, showing favourable tissue maturation of bioprinted MSCs [356, 357]. According to this framework, as crosslinks are cleaved, the network becomes more deformable and its equilibrium swelling ratio rises, accelerating degradation through greater solvent accessibility. This behaviour likely reflects a reduction in effective crosslink density, which facilitates both solvent diffusion and polymer chain mobility—processes typically associated with early-stage degradation. These experimental results align with theoretical predictions described by Pan *et al* (2022), who demonstrated through constitutive modelling that hydrolytic degradation in hydrogels intrinsically couples increased swelling and subsequent mass loss [404]. Therefore, the progressive increase in swelling observed in XG-rich formulations may indicate a more degradable and dynamically evolving support bath, consistent with the degradation–swelling–mass loss interplay described by Pan *et al.* (2022) [404]. Notably, deviations from the expected trend may also arise from experimental limitations, including variability in crosslinking efficiency, heterogeneity in network formation, and the influence of culture conditions on hydrogel stability. This tunability is particularly relevant for scaffold-free bioprinting, where excessive stability of the support bath can hinder post-printing fusion of cellular building blocks. The observed increase in swelling and mass loss with higher XG ratios suggests that physical entanglement between the two polymer networks remains sufficient to preserve short-term stability, while the unmodified component promotes hydrolytic susceptibility during long-term culture. Importantly, all formulations supported high cell viability and robust (fibro)chondrogenesis.

In bioprinting strategies for TE and regenerative medicine, tissue-specific cells or stem cells are commonly employed, with the expectation that they will differentiate and mature into the desired cell type to generate a biomimetic tissue. Musculoskeletal tissues, however, exhibit highly specific ECM organisation, particularly with respect to collagen fibre alignment, which is tightly coupled to their mechanical function. As a result, producing bioprinted tissues that are both anisotropic and mechanically functional remains a considerable challenge. Previous studies have applied developmental engineering concepts to guide the self-assembly [177] and self-organisation [64, 165] of μ Ts. These approaches, however, have largely relied on scaffold-based strategies, which can be associated with

prolonged *in vivo* degradation times. Furthermore, because 3D bioprinting is often intended to reproduce patient-specific defect geometries, the limited versatility of such scaffold designs can make it difficult to generate truly personalised constructs. This motivates alternative strategies to engineer anisotropic tissues in which the structural support that guides anisotropy and cell alignment is also removable and capable of directing differentiation prior to implantation. In this context, the ability of XG: XG-MA-based support baths to promote chondrogenesis and drive collagen alignment was assessed. Histological and biochemical results collectively indicate that neither the inclusion nor the increasing proportion of unmodified XG adversely affected the microenvironment or induced phenotypic drift. These findings underscore that the semi-IPN approach enables the introduction of degradability without altering the biochemical cues that sustain tissue differentiation.

Despite the overall equivalence in ECM composition, subtle structural differences emerged in collagen fibre alignment, particularly at higher XG ratios. The reduced birefringence intensity and coherency observed in the 3:1 formulation suggest that excessive degradation may disrupt the mechanical boundary conditions that normally guide ECM alignment during maturation. These results emphasise that, while degradability is essential for eventual tissue fusion, premature relaxation of the bath may reduce directional stress transfer, leading to locally disorganised collagen networks. The 1:1 ratio effectively balanced these opposing effects, preserving anisotropy while allowing progressive material clearance and filament fusion. At the macroscopic scale, the partially sacrificial behaviour of the XG: XG-MA blend enabled adjacent filaments to merge as the bath degraded, forming continuous and cohesive tissues without compromising alignment. This controlled fusion mimics the morphogenetic process of tissue compaction, where individual cellular modules fuse into mechanically unified structures [81, 181, 188, 347, 373]. The contrast between the XG-MA-only constructs, which retained discrete filament boundaries, and the blended system, which achieved structural integration, highlights the functional significance of partial degradability in scaffold-free printing. The collagen fibre orientation peaks and comparable coherency values between groups indicate that anisotropic organisation was maintained throughout this process.

Mechanical testing was consistent with these structural findings. All constructs displayed anisotropic tensile behaviour, indicating that the prescribed filament architecture was successfully translated into functional tissue organisation. The increased transverse stiffness observed in the XG: XG-MA constructs relative to the XG-MA-only group suggests improved load transfer between filaments, confirming that enhanced fusion leads to greater mechanical integration while preserving directional dependence, in line with the tensile behaviour of the native meniscus driven by oriented collagen fibres. Although the 1:1 blend improved the mechanical properties of the engineered tissue, the measured tensile properties remained well below reported average values for the human meniscus (60–200 MPa) [390]. Further enhancement of mechanical properties could potentially be achieved through additional interventions, such as chondroitinase ABC or lysyl oxidase treatment, which have been shown to increase the tensile properties of cartilage tissues [405-407]. Nevertheless, the results obtained here recapitulate key aspects of the hierarchical architecture of fibrocartilaginous tissues, in which local collagen anisotropy is coupled to emergent, directionally dependent mechanical function in the engineered constructs.

Collectively, these findings position the semi-IPN system as a versatile support bath for embedded bioprinting of scaffold-free, anisotropic tissues. By decoupling rheological stability from long-term persistence, the XG: XG-MA blend resolves a central limitation in traditional support-bath design: the inability to remove non-degradable materials without disrupting tissue integrity. This strategy could be expanded to other polysaccharide-based systems, enabling the production of larger, mechanically robust constructs that can eventually be cultured free of exogenous scaffolds. Future work should focus on refining the degradation kinetics to achieve full clearance of the support material within biologically relevant timeframes and on validating the *in vivo* biocompatibility of residual degradation products. Integrating this degradable system with dynamic culture conditions or mechanical stimulation could further enhance ECM organisation and functional maturation [408-413]. Ultimately, the results presented here demonstrated that controlled sacrificial behaviour in support baths can serve as a powerful design parameter to reconcile the structural and biological demands of scaffold-free bioprinting, advancing the generation of clinically relevant musculoskeletal grafts.

6.5. Conclusion

This work introduces a semi-IPN composed of XG and XG-MA as a degradable support bath for embedded bioprinting. The system preserves the rheological performance and print fidelity generated using the original XG-MA only support bath, while enabling controlled degradation, thereby expanding the functional window between structural stability and tissue fusion. The incorporation of unmodified xanthan modulated swelling and mass loss without compromising cell viability, chondrogenic differentiation, or ECM production. Importantly, the optimised 1:1 XG: XG-MA formulation supported progressive filament fusion and the formation of continuous, anisotropic tissues with enhanced structural integration. The scaled-up constructs maintained directional collagen alignment and displayed anisotropic mechanical behaviour consistent with their designed architecture. These findings establish degradability as a key tuneable parameter in support-bath design and demonstrate that dynamic, partially sacrificial systems can guide the maturation of scaffold-free, anisotropic grafts. The semi-IPN strategy presented here provides a scalable framework for engineering musculoskeletal tissues and may serve as a versatile platform for other biofabrication applications requiring both precision and adaptability.

Chapter VII: Region-Specific Growth Factor Cues to Bioprint Scaled-Up, Zonal, and Anisotropic Meniscal Tissues

7.1 Introduction

Meniscal injuries are a major cause of degenerative changes in the knee joint and are strongly associated with the onset of OA [414-416]. Since the meniscus plays a critical role in load distribution and joint stability, its impairment can lead to significant functional limitations, even during low-impact activities such as walking[417, 418]. However, the regenerative capacity of the meniscus is very limited, which greatly increases the risk of osteoarthritis following injury[363]. Current clinical treatments, including partial meniscectomy, meniscal allograft transplantation (MAT), remain the gold standards for managing meniscal injuries [419-421]. Nevertheless, their long-term outcomes are often unsatisfactory, as these strategies fail to fully restore the native meniscal structure and function. This limitation has driven growing interest in musculoskeletal TE approaches to develop biomimetic meniscal replacements.

The meniscus is a crescent-shaped fibrocartilaginous tissue composed of two distinct zones: an inner avascular region, rich in sGAGs and type II collagen, and an outer vascularized region, characterised by lower sGAGs and a higher content of type I collagen[68, 421, 422]. Overall, the tissue is predominantly composed of type I collagen, which is organised in a highly specific manner, with both circumferential and radial fibre alignment, conferring strong anisotropy to the meniscus[59, 423]. Therefore, engineering functional meniscal grafts will require recapitulating both the zonal biochemical composition and anisotropic architecture of the ECM of the native tissue [424]. A major challenge in meniscal TE is the development of grafts that replicate the native tissue's heterogeneity, including its specific sGAG and collagen content as well as their spatial organisation. Previous approaches have relied on meniscus-derived cells, enzymatically isolated from the native tissue and either seeded into scaffolds or hydrogels or allowed to self-assemble to generate meniscal constructs[425-427]. Yet, because the meniscus is organised into functionally distinct zones, a single cell source might be insufficient to replicate its structural and biochemical diversity. Our previous work demonstrated that region-specific fibrocartilage grafts can be

engineered by bioassembling two distinct populations of meniscus progenitor cells (MPCs)—one derived from the inner zone and one from the outer zone. These progenitor cells represent a promising source for engineering zonal grafts, as each cell type can be directed toward its respective regional phenotype [78]. However, isolating enough of these cells from a patient's defect, expanding them *in vitro*, and generating a patient-specific graft remains technically challenging and practically different to translate into a clinical setting. MSCs offer a more clinically feasible alternative due to their easier accessibility and multipotency [425, 428]. MSCs can be readily isolated from various human tissues (i.e. bone marrow, adipose tissue) and have emerged as a powerful tool in regenerative medicine [429].

Multiple strategies have been explored for meniscus regeneration, spanning scaffold-based to scaffold-free approaches. For example, partial repair has been achieved by seeding meniscus-derived cells onto biodegradable polyglycolic acid (PGA) scaffolds, though further long-term *in vivo* studies are needed to confirm structural stability and mechanical performance under joint loading [155, 430]. More recent approaches have aimed to mimic the zonal architecture of the native meniscus. Poly(lactic-co-glycolic acid) (PLGA) microparticles have been used for region-specific delivery of connective tissue growth factor (CTGF) to stimulate outer zone development and transforming growth factor beta-3 (TGF- β 3) for inner zone regeneration. When incorporated into polycaprolactone (PCL)-reinforced scaffolds, these systems have shown promise in preclinical models. By enriching scaffolds with PLGA microparticles loaded with CTGF and TGF- β 3, researchers have successfully engineered grafts with zonally distinct features, recapitulating the collagen distribution and the zonal organisation of native meniscus [155]. In native tissue, CTGF expression is mechanically regulated and promotes collagen I production, while co-delivery of CTGF and TGF- β 3 has been shown to induce a fibrocartilaginous phenotype characterised by the presence of both collagen I and II [154, 431]. These growth factors can be incorporated into bioinks to enable controlled local release during bioprinting. Nanosilicates such as Laponite represent a versatile tool in this context, as they have been widely applied in TE and 3D bioprinting [432, 433]. Under shear, their disc-like shape and orientation can enhance both the mechanical and biological performance of bioprinted constructs [434]. Owing to their positive surface charge, Laponite nanosilicates can readily

bind growth factors and have been leveraged to develop composite hydrogels for 3D bioprinting with controlled release profiles [435]. For example, Laponite has been used to modulate VEGF release in an osteochondral graft model, where its incorporation into a methylcellulose-based bioink (slow-release) significantly delayed VEGF release compared to an otherwise identical bioink lacking Laponite (fast-release) [138]. Building on this, it was hypothesised that Laponite could be used to control the release of TGF- β and CTGF for meniscal 3D bioprinting applications. By combining such growth factor-loaded bioinks with precise spatial deposition, it should be possible to generate tissues with an outer region enriched in collagen I and an inner region enriched in collagen II. However, the above-mentioned strategies are all scaffold-based and might not be able to fully replicate a biomimetic tissue, and the limitations of the material when implanted *in vivo* might promote a foreign body response and impair the tissue regeneration

Bottom-up tissue engineering is a relatively new paradigm that relies on assembling cellular building blocks—such as cell sheets, spheroids, or μ Ts—to generate larger constructs. μ Ts are particularly advantageous because they act as single, functional units that can be assembled without the need for exogenous scaffolds, thereby avoiding complications associated with foreign biomaterials[36, 237]. Their self-assembly and self-organisation properties make them especially useful in musculoskeletal TE, where these processes are critical for functional tissue development [436-438]. In this study, the μ Ts were bioprinted into a gelatin/Laponite-based bioink within a XG-MA support bath for meniscal TE. Laponite was introduced as a delivery platform for controlled, spatial release of CTGF and TGF- β 3, thereby enabling the generation of zonally defined meniscal grafts.

The experimental design involves first fusing μ Ts (without the use of any supporting hydrogel) and subjecting them to different growth factor regimes, specifically CTGF and/or TGF- β 3 stimulation. The most promising combinations will then be bioprinted in simple filament patterns using gelatin and laponite loaded with different growth factor regimes, to assess the final phenotype of each filament. Ultimately, dual-cartridge bioprinting was employed: one bioink loaded with TGF- β 3 for the inner zone and another with CTGF and TGF- β 3 for the outer zone, producing scaled-up, region-specific fibrocartilage grafts. This strategy aims to bridge the gap between generating larger grafts and achieving precise zonal organisation in a scaffold-free meniscal construct.

7.2. Materials and methods

7.2.1 Isolation and expansion of gBM-MSCs and μ Ts formation

Caprine bone marrow MSCs were isolated from the sternal marrow of skeletally mature female goats. Marrow fragments were minced and vortexed in high-glucose DMEM (Gibco) with 10% FBS and penicillin/streptomycin (XPAN) to release cells. At ~80% confluence, cells were cryopreserved in liquid nitrogen. For experiments, MSCs were reseeded at 5×10^3 cells·cm⁻² under 5% O₂ and expanded to passage 3 (P3).

For μ Ts formation, 3D-printed mould stamps (1,889 recesses) and 4% (w/v) ultrapure agarose (Sigma) were autoclaved at 120 °C for 20 min. Agarose was cast into 6-well plates, moulded, and cooled for 15 min, then soaked overnight in XPAN at 37 °C under physioxenic conditions (5% O₂, humidified atmosphere). MSCs were seeded into the microwells at 7.556×10^5 cells·mL⁻¹ (5 mL per well), centrifuged at 700 × g to collect cells into the recesses, and yielded 1,889 μ Ts with ~2,000 cells per μ T. μ Ts were cultured in basal chondrogenic media (CDM-) (hgDMEM GlutaMAX supplemented with penicillin/streptomycin, sodium pyruvate 100 μ g·mL⁻¹, L-proline 40 μ g·mL⁻¹, L-ascorbic acid-2-phosphate 50 μ g·mL⁻¹, linoleic acid 4.7 μ g·mL⁻¹, bovine serum albumin 1.5 mg·mL⁻¹, 1× ITS, and dexamethasone 100 nM; all Sigma). After 2 days, μ Ts were harvested; suspension density was quantified and the volume adjusted to reach the desired concentration for each experiment.

7.2.2 μ T fusion and fibrochondrogenic differentiation of fused μ Ts

On day 2, 12-well plates were coated with 2% (w/v) sterile, autoclaved agarose and equilibrated with XPAN for 1 h. For each condition, 150 μ Ts were seeded per well and incubated at 37 °C for 1 h to pre-fuse before gently adding medium. Cultures were maintained for 4 weeks in CDM- basal medium with the following regimens: (1) TGF- β 3, 10 ng/mL (4 weeks); (2) CTGF, 100 ng/mL (4 weeks); (3) TGF- β 3 → CTGF, 10 ng/mL TGF- β 3 for 2 weeks followed by 100 ng/mL CTGF for 2 weeks; (4) CTGF → TGF- β 3, 100 ng/mL CTGF for 2 weeks followed by 10 ng/mL TGF- β 3 for 2 weeks; (5) CTGF + TGF- β 3, 100 ng/mL CTGF plus 10 ng/mL TGF- β 3 for 4 weeks. Medium was changed three times per week.

7.2.3 Support bath (XG-MA) and bioink synthesis (Laponite/gelatin)

XG (0.5% w/v) in ultrapure water was reacted with glycidyl methacrylate (40 mL) overnight at 60 °C in the dark, dialysed 7 days (twice-daily water changes), lyophilised 48 h, stored at –20 °C, and EO-sterilised (12 h). Two days before printing, XG-MA was dissolved in phenol red-free DMEM with penicillin (100 U/mL) and streptomycin (100 µg/mL) and rotated at 40 rpm at room temperature. On the print day, LAP was added (0.25% w/v), the bath was centrifuged to de-gas ($2,500 \times g$, 5 min, RT), and 4 mL was dispensed into a 6-well plate using a positive-displacement pipette.

For the bioink preparation, Laponite XLG (BYK Additives & Instruments, UK) was autoclaved at 120 °C for 20 min and subsequently dried to remove residual moisture. DMEM was then added, and the suspension was stirred at 700 rpm for 2 days on a magnetic stir plate. The Laponite dispersion was combined with a sterile gelatin solution and the appropriate growth factor, then stirred overnight at room temperature to promote growth factor binding to the nano clay and to obtain a homogeneous mixture. On the day of printing, the bioink was chilled at 4 °C to promote complete gelation and then mixed with μ Ts to yield final concentrations of 1% (w/v) gelatin and 0.05% (w/v) Laponite and 45,000 μ Ts/mL. The total growth factor load was calculated as the cumulative dose for a 4-week study, assuming 250 mL of medium: 25 µg for CTGF and 2.5 µg for TGF- β 3.

7.2.4 Rheological analysis of bioinks

Rheological tests were performed on a MCR 102 rheometer (Anton Paar) with Peltier temperature control using a 25 mm parallel-plate geometry (PP25). The flow curve (viscosity vs shear rate, $0.1\text{--}1000\text{ s}^{-1}$) was acquired at 4°C for laponite/gelatin-based bioinks. Samples were maintained in a high-humidity environment to prevent dehydration. All measurements were performed in triplicate.

7.2.5 Growth factor release from bioprinted filaments in media and release quantification over time

To evaluate 4-week release profiles of CTGF and TGF- β 3 bound to Laponite, bioinks loaded with either factor were printed into a 1% XG-MA support bath and UV-crosslinked for 4 min (as described previously). For each well of a 6-well plate, eight 10 mm filaments were

printed, corresponding to a theoretical load of 0.8 μg TGF- β 3 per well or 8 μg CTGF per well. Each condition was printed in triplicate. Constructs were cultured for 28 days; medium was sampled twice per week and snap-frozen at -70°C . Growth factor concentrations in the collected media were quantified by ELISA for CTGF and TGF- β 3 (Bio-Techne), following the manufacturer's protocol, with absorbance read at 450 nm on a microplate reader. For analysis, samples collected within the same nominal interval were pooled and assigned to the terminal day of that interval (e.g., days 2, 5, and 7 pooled and reported as day 7).

7.2.6 Bioprinting of μT s and dual cartridge bioprinting set-up

For bioprinting, μT s were harvested on day 2 and mixed with the designated bioinks. For single-filament prints, μT s were combined with three formulations: (1) TGF- β 3-loaded bioink (2.5 μg), (2) dual-loaded bioink containing CTGF and TGF- β 3 (25 μg and 2.5 μg , respectively), and (3) a non-loaded bioink, with the growth factor supplied in the culture medium changed three times per week. For prints in which the growth factor was incorporated into the ink, the culture medium was basal CDM-. For dual-cartridge prints. Two cartridges were used: a "chondro-ink" (TGF- β 3, 2.5 μg) and a "fibro-ink" (CTGF plus TGF- β 3). Both syringes were prepared, loaded into the bioprinter (CELLINK), and calibrated to the same origin. For the meniscus construct, three half-circumferential lines were printed for the outer region and two lines for the inner region. Printing employed a piston-based syringe-pump printhead with an extrusion rate of 6 $\mu\text{L/s}$ and a print speed of 2 mm/s.

7.2.7 Histological analysis

For construct analysis, constructs were retrieved by mechanically disrupting the surrounding support bath and carefully extracting the samples using tweezers. Briefly, samples were fixed in 4% paraformaldehyde (PFA) overnight at 4°C . After fixation, they were dehydrated through a graded ethanol series (50–100% v/v), cleared in xylene, and embedded in paraffin wax (Sigma) as previously described. Sections of 5 μm thickness were cut, rehydrated, and subjected to histological staining. Haematoxylin and eosin (H&E; Sigma) was used to assess tissue morphology, ECM, and nuclei. sGAG content was stained with 1% (w/v) Alcian Blue (AB) 8GX in 0.1 M HCl and counterstained with 0.1% (w/v) nuclear fast red for cell distribution. Collagen deposition was visualised using 0.1% (w/v) Picrosirius

Red (PR; Sigma). Stained sections were mounted in Pertex (Avantor) and imaged with an Aperio ScanScope slide scanner (Leica, Germany).

7.2.8 Biochemical evaluation

After retrieval, the samples were washed twice with 1X PBS and dried. Papain enzyme solution created with 3.88 U/mL of papain enzyme, 100 mM sodium phosphate buffer, 5 mM Na₂EDTA, and 10 mM L-cysteine at pH 6.5 (all from Sigma), was then added to digest the samples at 60°C for 18 hours. Immediately after digestion, DNA content was quantified using the Quant-iT™ PicoGreen® dsDNA Reagent and Kit (Molecular Probes, Biosciences). The amount of sulphated glycosaminoglycan (sGAG) was determined using a dimethylmethylene blue dye (DMMB, Sigma) binding assay. To exclude any background absorbance, the pH of the DMMB was adjusted to 1.5. A chondroitin sulphate standard (1 mg/mL, Sigma) was used, and the absorbance was measured at 530 and 590 nm using a Synergy HT multi-detection microplate reader (BioTek Instruments, Inc). The 530/590 absorbance ratio was used to generate the standard curve and determine the sGAG concentration from the digested sample. Total collagen content was determined by measuring the hydroxyproline content using a chloramine-T assay. The samples were mixed with 38% HCl (Sigma) (1:1 ratio) and incubated at 110°C for 18 hours for hydrolysis. After cooling, samples were centrifuged at 5000 x g for 5 minutes, and let dry at 60°C for 48 h. The sediment was reconstituted in 200 µL of ultra-pure H₂O. Chloramine T (2.82% w/v) and 4-(Dimethylamino) benzaldehyde (0.05% w/v) were added and incubated for 20 minutes at RT in the dark. The hydroxyproline content was quantified using a trans-4-Hydroxy-L-proline standard at a wavelength of 570 nm with a Synergy HT multi-detection microplate reader (BioTek Instruments, Inc). Hydroxyproline levels were estimated from the standard curve at a wavelength of 570 nm. The hydroxyproline-to-collagen ratio of 1:7.69 was used to calculate the collagen content.

7.2.9 RT-qPCR analysis of bioprinted constructs

At day 28 post-bioprinting, the samples were washed 3 times with 1X PBS and then snap frozen for further processing. RNA was isolated from the samples using the Trizol method (Sigma-Aldrich), following the manufacturer's instructions. Chloroform extraction was performed, and the RNA was resuspended in RNase-free water. The RNA samples were then

stored at -80°C. For cDNA synthesis, a High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™), was used to transcribe 500 ng of RNA from each sample into cDNA (20 µL) via polymerase chain reaction (PCR). Real-time PCR was performed using an Applied Biosystems instrument, and Taqman PCR universal master mix (ThermoFisher) was used for the PCR reaction. Human-specific TaqMan probes (ThermoFisher) were used for gene amplification. The qPCR amplification followed the following cycles: 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles at 60°C for 1 minute each. The levels of gene expression were quantified using the real-time PCR data and analysed with the $2(-\Delta\Delta C_t)$ method. The expression levels were normalised to the average expression of the 18S rRNA housekeeping gene. All experiments were performed in triplicate.

7.2.10 Live/Dead staining

Cell viability was assessed using a live/dead assay kit (Invitrogen, Bioscience). The constructs were first rinsed in PBS 1X and then incubated for 1 hour in a solution containing 2 µM of calcein and 4 µM ethidium homodimer-1 (EthD-1). Following the incubation period, the constructs were rinsed again to remove any excess of the dyes and were imaged using a Leica SP8 scanning confocal microscope. Excitation wavelengths of 485 nm and 530 nm were used for calcein, while emission wavelengths of 530 nm and 645 nm were used for calcein and EthD-1. Maximum projection z-stack reconstructions were generated to analyse cell viability throughout the depth of the tissue, with the view from the top.

7.2.11 Immunofluorescence evaluation

Following fixation in 4% paraformaldehyde overnight and paraffin processing (Section 6.2.5), sections were rehydrated through graded ethanol (100–50%) into 1× PBS (Gibco). Antigen retrieval was performed enzymatically with Hyaluronidase (4,000 U/mL, 25 min, 37 °C; Sigma-Aldrich) followed by Pronase (3.5 U/mL, 25 min, 37 °C, Sigma). Sections were rinsed and blocked for 1 h at room temperature in PBS containing 1% BSA and 10% goat serum. Primary antibodies to collagen I (Abcam, ab90395; 1:400) and collagen II (Santa Cruz, sc-52658; 1:400) were applied in blocking buffer and incubated overnight at 4 °C in a humidified chamber. After three PBS washes, appropriate secondary antibodies in 2% BSA were applied for 1 h at room temperature in the dark. Slides were washed, mounted with Fluoroshield containing DAPI (Sigma), sealed, and stored at 4 °C in the dark. Imaging was

performed on a Leica SP8 confocal microscope; representative images are shown as maximum-intensity projections of z-stacks.

7.2.12 Image quantification analysis of immunofluorescence pictures and PLM analysis

For immunofluorescence quantification, the intensity of 3 specific regions of interest (ROI) within each sample was measured and quantified through ImageJ software. The intensity was then normalised by the number of nuclei in the ROI. For PLM analysis, Sections were stained with 0.1% (w/v) Picrosirius Red, mounted with DPX (Sigma-Aldrich), and imaged on an Olympus BX41 polarising light microscope equipped with a MicroPublisher 6 CCD camera and an Olympus U-CMAD3 adapter. Collagen fibril orientation and coherency were quantified in ImageJ using the OrientationJ and Directionality plugins.

7.3 Results

7.3.1 MSC μ T fusion and fibrocartilaginous development under altered growth factor stimulation regimes

Scaffold-free fabrication of fibrocartilaginous grafts by fusing multiple μ Ts requires both large μ T numbers and conditions that favour a fibrocartilage over a hyaline-like cartilage phenotype. The primary objective was to produce fibrocartilage μ Ts at scale and fuse them into larger grafts. As nutrient transport limitations are a concern when fusing a large number of μ Ts (as shown in Chapter III), we first stimulated relatively small numbers of μ Ts (150 μ Ts per construct; 2,000 cells/ μ T; 3×10^5 cells/construct) to the different growth factor regimens (Fig. 7.1A). Day 2 μ Ts were harvested and seeded into an agarose mould at uniform density (Fig. 7.1 A–G). Fusion was evident within 24 h, and over 21 days, the μ Ts formed compact tissues (Fig. 7.1 B).

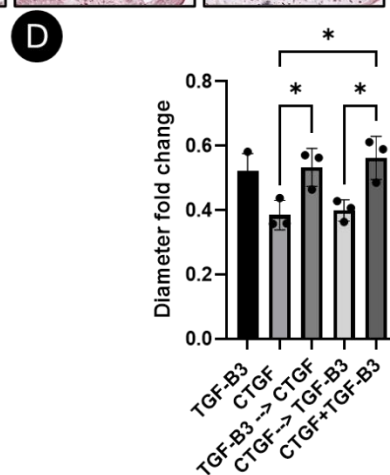
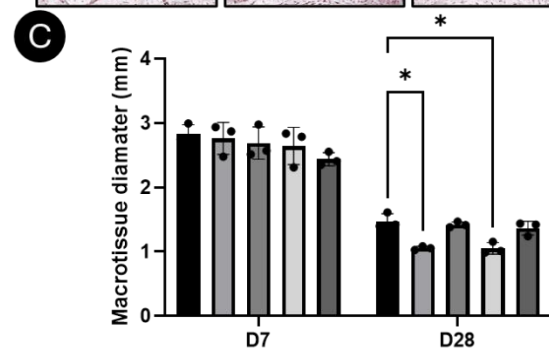
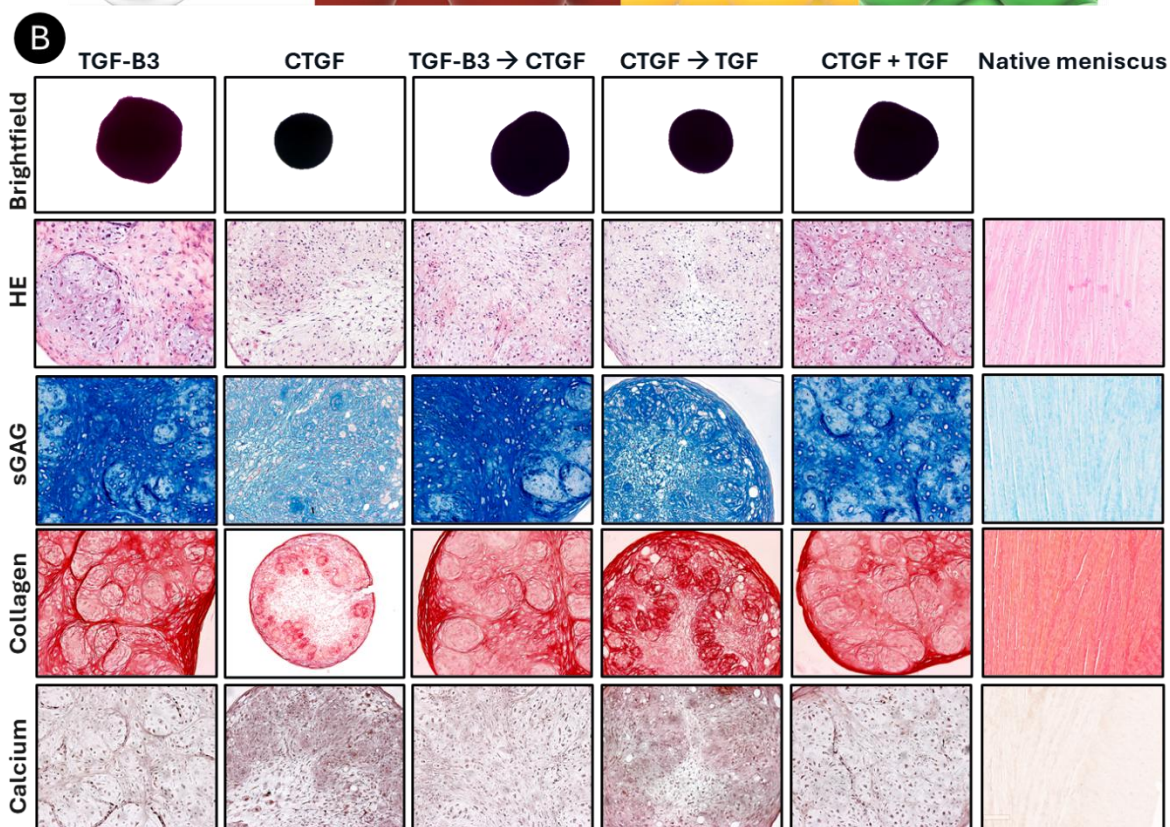
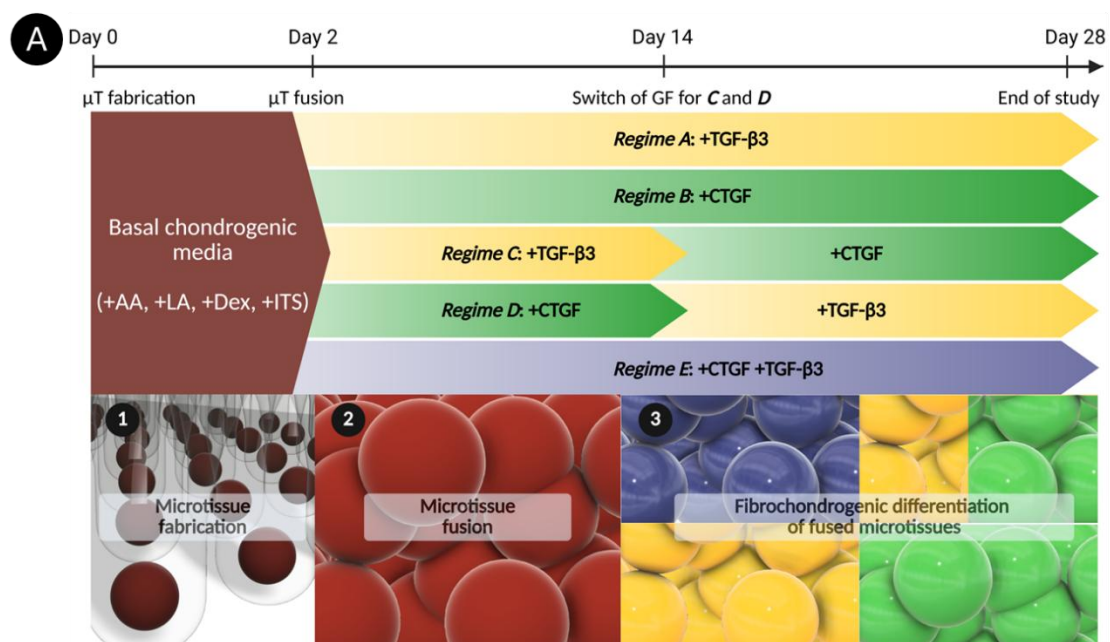


Figure 7.1. MSCs μ Ts show distinct patterns of growth and matrix deposition after 28 days in culture under different growth factor regimes. (A) Schematic of the growth factor regimens applied during μ T fusion. μ Ts were fused at day 2 post formation, and the different regimes were applied. At day 14, the growth factors were switched in regimes C and D. At day 28, the study was stopped and the samples analysed. Step 1: μ T fabrication; step 2: μ T fusion; step 3: differentiation of fused μ Ts. (B) Brightfield and histological assessment of fused μ Ts after 28 days under the following regimens: TGF- β 3 for 4 weeks; CTGF for 4 weeks; TGF- β 3 for 2 weeks followed by CTGF for 2 weeks; CTGF for 2 weeks followed by TGF- β 3 for 2 weeks; and combined TGF- β 3 + CTGF for 4 weeks. Histological stains: H&E (morphology), Alcian Blue (sGAG), Picrosirius Red (total collagen), and Alizarin Red (calcium deposit/mineralisation) (C) μ T diameter at day 7 (D7) and day 28 (D28). (D) Diameter fold change across regimens, defined as D28/D1. N=3, significant differences were determined using a two ANOVA followed by a Tukey's test post hoc comparison for (C) and an ordinary one-way ANOVA followed by a Tukey's multiple comparison test for (D).

Fusion of μ Ts occurred for all growth-factor regimens. By day 28, construct size differed by regimen, with a large standard deviation in groups treated with CTGF for the full 4 weeks and those primed with CTGF for 2 weeks followed by TGF- β 3 (CTGF $\rightarrow$ TGF- β 3) for the subsequent 2 weeks (Fig. 7.1 C, D). Histology demonstrated regimen-dependent sGAG and collagen deposition (Fig. 7.1 B). Continuous TGF- β 3 exposure for 4 weeks, as well as TGF- β 3 $\rightarrow$ CTGF stimulation, supported greater sGAG deposition. CTGF $\rightarrow$ TGF- β 3 stimulation did not appreciably enhance sGAG accumulation, and CTGF alone failed to support appreciable sGAG and collagen deposition (Fig. 7.1 B). Calcium staining was negligible relative to the native meniscus, indicating no mineralisation of the resulting tissues (Fig. 7.1 B).

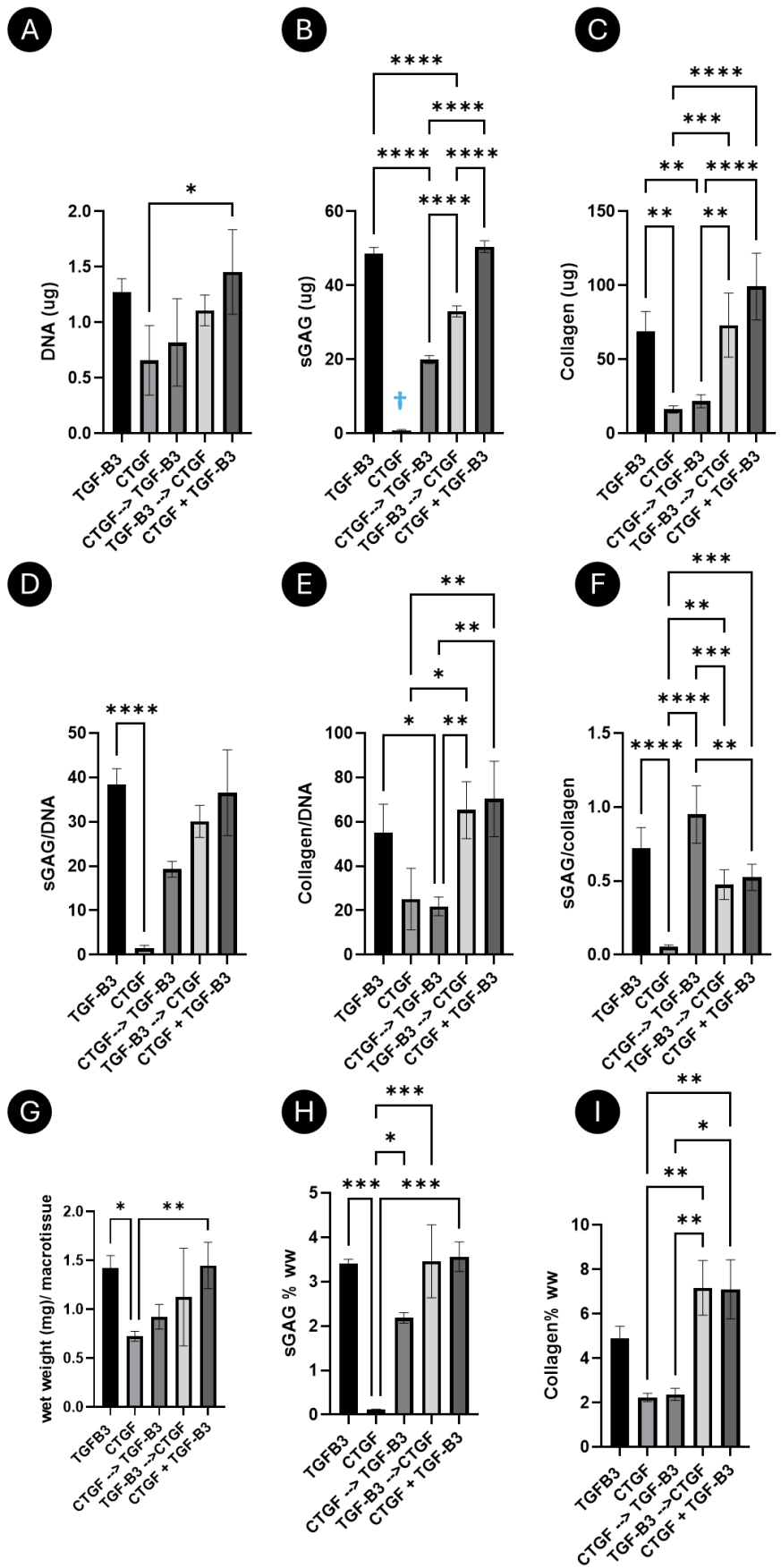


Figure 7.2. Self-assembled μ Ts show distinct patterns of growth and matrix deposition after 28 days in culture under different growth factor regimes. (A) DNA quantification revealed differences in cellularity, with the CTGF+TGF- β 3 group containing significantly more cells than CTGF alone. (B) sGAG accumulation was highest in groups exposed to TGF- β 3, particularly in the TGF- β 3 and CTGF+TGF- β 3 groups, whereas sequential treatments resulted in reduced deposition. (C) Collagen content followed a similar trend, with greater accumulation when TGF- β 3 was present during the early phase of culture, and a modest increase in the CTGF+TGF- β 3 group compared to TGF- β 3 alone. (D-E) When normalised to DNA, both sGAG and collagen deposition confirmed these differences, highlighting the importance of growth factor regime in regulating matrix biosynthesis. (F) The sGAG-to-collagen ratio further emphasised the distinct balance between cartilaginous and fibrous matrix production across groups. (G) Wet weight measurements showed comparable mass in TGF- β 3 and CTGF+TGF- β 3 groups, whereas CTGF constructs were significantly smaller. (H-I) Normalisation of matrix components to wet weight revealed that TGF- β 3 induced the highest sGAG percentage, while collagen percentage was greatest in the TGF- β 3 $\rightarrow$ CTGF and CTGF+TGF- β 3 groups, though values remained below native meniscus levels. N=4, significant differences were determined using an ordinary one-way ANOVA followed by a Tukey's multiple comparison test, where * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, and **** denotes $p < 0.0001$.

Neo-tissue growth and maturation were evaluated after 4 weeks of chondrogenic culture under different growth factor regimes (Fig. 7.2). DNA content differed significantly between the CTGF and CTGF+TGF- β 3 groups ($p < 0.05$) (Fig. 7.2 A). After 28 days, all groups containing TGF- β 3 exhibited higher sGAG deposition, with the greatest accumulation observed in TGF- β 3 and CTGF+TGF- β 3 groups (Fig. 7.2 B). This trend was maintained when sGAG levels were normalised to DNA (Fig. 7.2 D). A similar pattern was observed for collagen, with increased levels in groups where TGF- β 3 was present during the first two weeks of induction. There was a trend towards higher collagen accumulation in the CTGF+TGF- β 3 group compared to TGF- β 3 alone ($p = 0.07$) (Fig. 7.2 E). By contrast, the CTGF and CTGF $\rightarrow$ TGF- β 3 groups contained significantly less collagen, underscoring the importance of TGF- β 3 during the initial induction phase for collagen biosynthesis (Fig. 7.2 C, E). Normalisation of collagen content to DNA yielded similar trends, with statistically significant differences maintained across groups. The sGAG/collagen ratio differed significantly across conditions (Fig. 7.2 F). TGF- β 3 stimulation resulted in a high ratio (~ 0.7), which was significantly greater than CTGF alone (~ 0.01 , $p < 0.0001$). Sequential stimulation with CTGF $\rightarrow$ TGF- β 3 yielded the highest ratio ($\sim 0.8-0.9$), significantly greater than TGF- β 3 alone ($p < 0.01$) and all other groups ($p < 0.0001$). In contrast, TGF- β 3 $\rightarrow$ CTGF (~ 0.5) and

CTGF+TGF- β 3 (~0.5) produced intermediate values, both significantly lower than TGF- β 3 ($p < 0.01$ – 0.001) and CTGF $\rightarrow$ TGF- β 3 ($p < 0.0001$) (Fig. 7.2 F). After 28 days, the wet weight of each construct was also measured (Fig. 7.2 G). The wet weight of constructs was comparable between TGF- β 3 and CTGF+TGF- β 3 groups, while it was significantly reduced in the CTGF group compared to both TGF- β 3 ($p < 0.05$) and CTGF+TGF- β 3 ($p < 0.01$) (Fig. 7.2 G). When normalised to wet weight, TGF- β 3 stimulation yielded the highest sGAG content (Fig. 7.2 H), although this value exceeded that typically observed in native meniscus and was more comparable to articular cartilage (Fig. 7.2 H). In contrast, normalisation of collagen to wet weight revealed that the TGF- β 3 $\rightarrow$ CTGF and CTGF+TGF- β 3 groups contained significantly higher collagen levels than CTGF and CTGF $\rightarrow$ TGF- β 3 ($p < 0.01$). Nevertheless, the maximum collagen content achieved (~8% of wet weight) remained below native meniscus values (~22%) [439]. These findings highlight the need for a trade-off between maintaining physiologically relevant levels of sGAG and increasing collagen deposition to approach the native tissue composition. Thus, tuning the growth factor regime allows modulation of matrix composition toward zonal biomimicry of the native meniscus.

7.3.2 Fused μ Ts develop distinct ECM in response to growth factors

After 28 days of culture under different growth factor regimes, fused μ Ts were analysed by immunofluorescence to assess ECM composition (Fig. 7.3). While sGAG and total collagen quantification reflected overall matrix deposition, true meniscus biomimicry requires reproduction of the tissue's zonal collagen composition. To evaluate this, we examined the deposition of collagen type I and II to determine not only which conditions supported

robust ECM formation but also which best replicated native zonal patterns. Distinct differences emerged between the groups (Fig. 7.3).

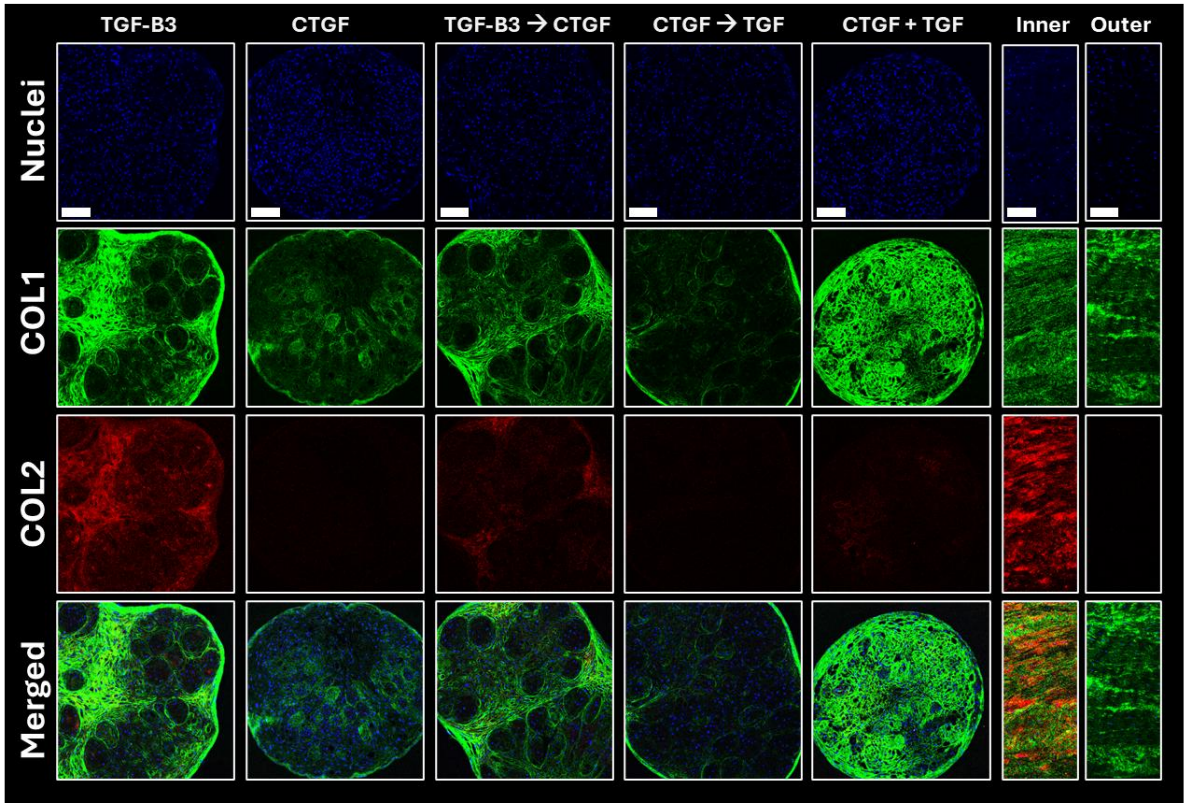


Figure 7.3. Microtissues develop a distinct pattern of ECM deposition when exposed to different growth factor regimes. μ Ts were harvested at 2 days post-fabrication and fused in a low-adherent agarose mould and kept in culture for 28 days at different growth factor regimes: 1) TGF- β 3 only for 4 weeks(TGF- β 3), 2)CTGF only for 4 weeks (CTGF), 3)TGF- β 3 for 2 weeks and switch to CTGF for the remaining two weeks (TGF- β 3→CTGF), 4) CTGF for 2 weeks and subsequently TGF- β 3 for the remaining 2 weeks (CTGF→TGF- β 3), 5) CTGF and TGF- β 3 together for 4 weeks (CTGF+TGF- β 3). At the end of the culture, the resultant grafts were stained against chondrogenic markers, collagen type I (COL1) and collagen type II (COL2). Scale bar: 200 μ m.

The CTGF+TGF- β 3 group showed the most intense staining for collagen I (Fig. 7.3). TGF- β 3 alone promoted strong collagen type II staining, consistent with a more hyaline cartilage-like phenotype (Fig. 3). In contrast, CTGF alone, CTGF→TGF- β 3, and TGF- β 3→CTGF groups exhibited weaker staining for both collagen types (Fig. 7.3). These findings suggest that combined CTGF+TGF- β 3 stimulation best supports an outer zone meniscus phenotype, with collagen type I predominating over collagen type II. Because collagen type II is normally

enriched in the inner meniscus, two groups were selected for subsequent bioprinting studies: TGF- β 3 alone to support an inner region phenotype rich in both collagen type I and II, and CTGF+TGF- β 3, to support an outer region phenotype with higher levels of collagen type I (Fig. 7.3).

7.3.3 Controlled release of growth factors from laponite functionalised bioinks

After identifying the most suitable growth factor regimens for promoting the zone-specific differentiation of meniscal μ T-based constructs, this thesis next sought to determine if such growth factors could be co-printed with μ Ts within a XG-MA support bath. The bioink formulation was based on the findings of the previous chapters, as was the choice of the support bath (Chapter IV). Because gelatin functions as a sacrificial bioink, loading growth factors directly into it would result in their rapid release within 24 hours, coinciding with gelatin dissolution, as previously demonstrated (Chapter IV). To enable controlled release over four weeks, an additional delivery system was required. Building on previous studies, Laponite was incorporated into a gelatin bioink to regulate the release of CTGF and TGF- β 3, leveraging the electrostatic interactions between positively charged Laponite and the predominantly negatively charged growth factors. This formulation—comprising laponite (0.05 v/w %), gelatin (1%), and μ Ts (45,000 μ Ts/mL) —was designated as the principal bioink for the study (Fig. 7.4 A)

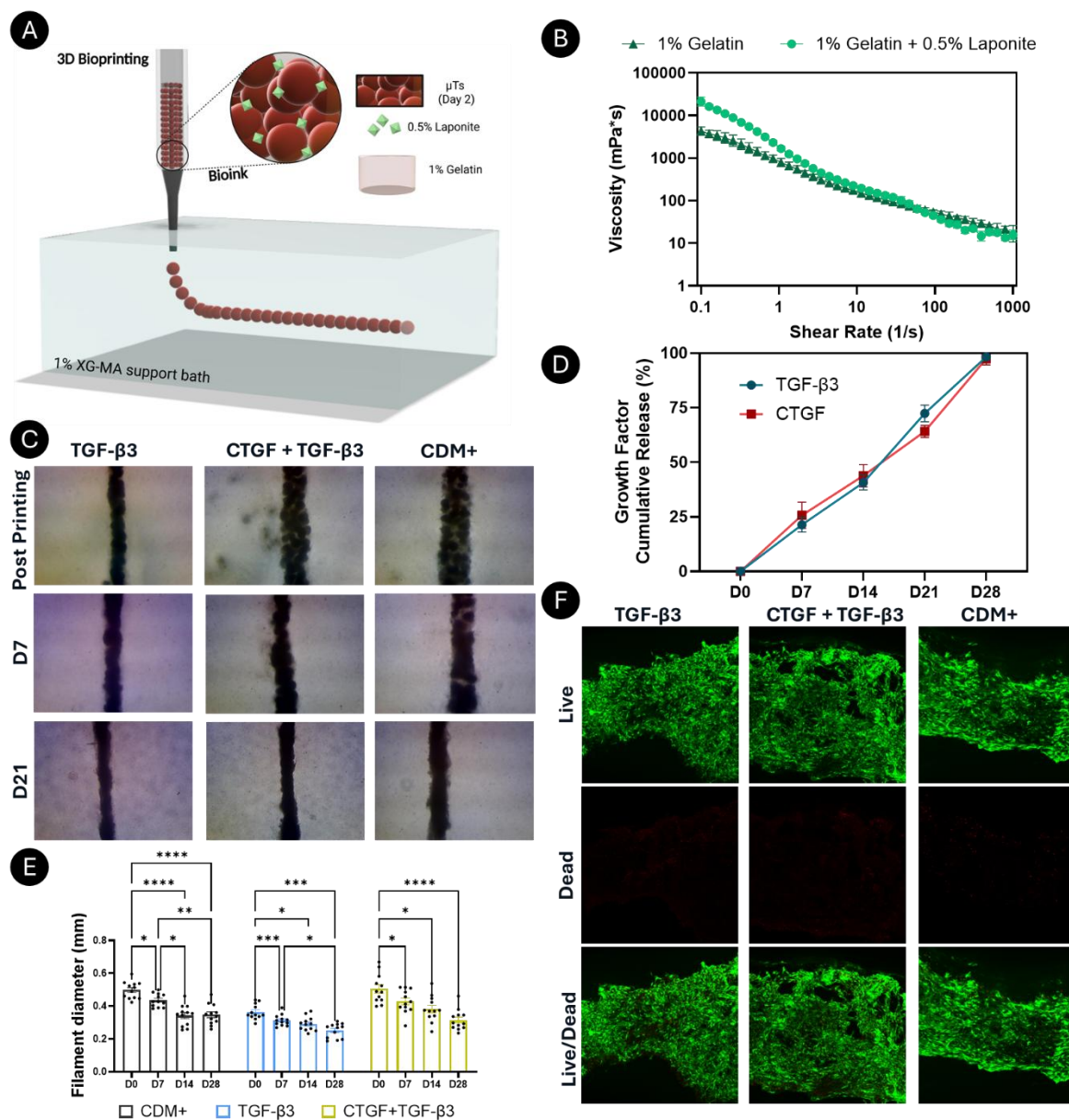


Figure 7.4. Localised and sustained delivery of growth factors in bioprinted μ Ts affects tissue compaction and cell viability. A) Schematic representation of the bioink formulation and printing of μ Ts at a density of 45,000 μ Ts/mL into an XG-MA support bath. B) Viscosity profiles of gelatin bioink and laponite+ gelatin-based bioink measured over a shear rate range of 0–1000 s^{-1} at 4 $^{\circ}\text{C}$ (N = 3). C) Brightfield microscopy images of printed μ Ts in fibro-ink supplemented with different growth factors over 21 days of culture, showing tissue compaction and μ T fusion. D) Release of growth factors from the bioinks assessed by ELISA (N = 3). E) Filament diameter of printed μ Ts in fibro-ink across different conditions (N = 12). F) Viability assessment of printed μ Ts in fibro-ink after 28 days in culture, post-printing. Significant differences were determined using one-way ANOVA followed by Tukey's multiple comparison test, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Printability of the two Laponite–gelatin bioink formulations was first evaluated by rheological assessment of their shear-thinning behaviour. Both formulations displayed favourable shear-thinning properties, with the Laponite-containing bioink exhibiting a higher initial viscosity (Fig. 7.4B). μ Ts were then bioprinted into the support bath using previously optimised parameters (6 μ L/s extrusion rate, 3 mm/s print speed) (Fig. 7.4 C). Three main groups were investigated: (1) a Laponite/gelatin bioink supplemented with TGF- β 3, hereafter referred to as the *chondro-ink*; (2) a Laponite/gelatin bioink supplemented with CTGF+TGF- β 3, referred to as the *fibro-ink*; and (3) a Laponite/gelatin bioink without added growth factors, where TGF- β 3 was supplemented directly into the media (CDM+). The incorporation of Laponite and growth factors did not adversely affect printability, as indicated by comparable filament diameters (Fig. 7.4 E) and rheological profiles relative to earlier formulations (Fig. 7.4 B). Filament diameter decreased over time in all conditions (Fig. 7.4 E). In the CDM group, diameters were initially $\sim$ 0.45–0.50 mm and progressively declined to $\sim$ 0.30 mm by day 28 ($p < 0.0001$). μ Ts printed with the chondro-ink generated filaments with slightly smaller diameters ($\sim$ 0.30–0.35 mm), with further reductions by day 28 ($p < 0.001$). Similarly, fibro-ink printed constructs decreased from $\sim$ 0.35–0.40 mm at baseline to $\sim$ 0.25–0.30 mm over the same period ($p < 0.001$). These consistent reductions likely reflect a combination of compaction imposed by the support bath and biologically driven remodelling, as growth factor release promoted μ T fusion and more homogeneous tissue formation (Fig. 7.4 C).

To characterise the release kinetics, we quantified the cumulative elution of CTGF and TGF- β 3 from the bioink formulations by ELISA at defined time points (Fig. 7.4 D). Medium collected 6 h after bioprinting (designated day 0) contained no detectable growth factors, confirming the absence of an initial burst release (Fig. 7.4 D). Thereafter, both factors were released in a sustained manner: cumulative release reached $\sim$ 20% by day 7, increased to $\sim$ 40–45% by day 14, and to $\sim$ 60–70% by day 21. By day 28, cumulative release was 97–100%, indicating near-complete release over the four weeks (Fig. 7.4 D). The profiles for CTGF and TGF- β 3 were comparable throughout, consistent with controlled, gradual delivery from the bioinks. Cell viability was evaluated after 28 days of culture under all conditions. High levels of viability were observed in all printed filaments, indicating that the inclusion

of laponite, sustained growth factor release, and the bioprinted microenvironment did not compromise cell survival (Fig. 7.4 D).

7.3.4 Co-printing of growth factors and μ Ts supports the development of fibrocartilage-like tissue

To investigate the effect of printed growth factor delivery on MSC phenotypical response, μ Ts were cultured for 28 days in CDM. In the CDM group, constructs were printed with laponite and gelatin without incorporated growth factors, but the medium was supplemented with 10 ng/ml of TGF- β 3 (Fig. 7.5).

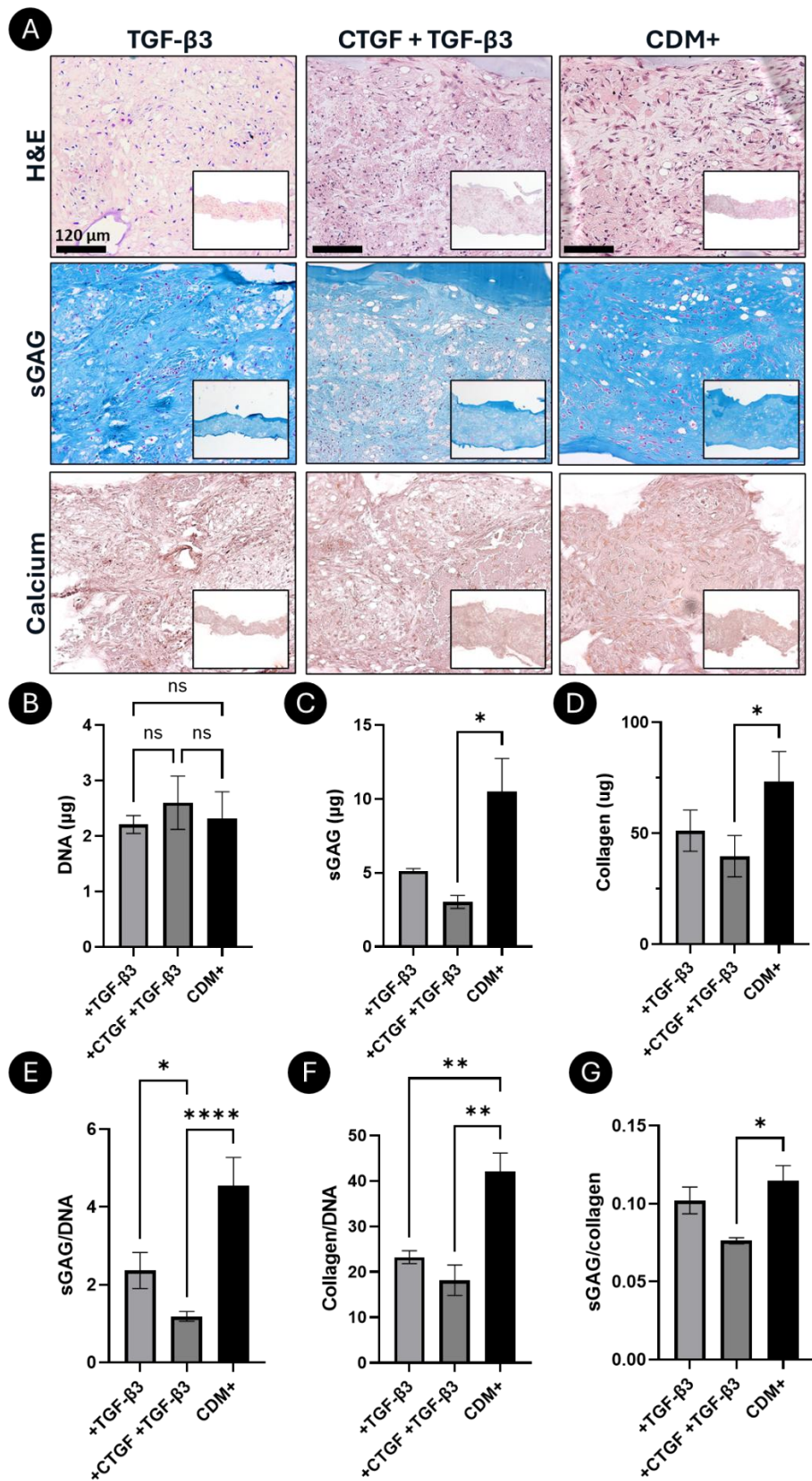


Figure 7.5. Printed μ Ts remodel their ECM toward a fibrocartilage phenotype within 28 days when encapsulated within a controlled release bioink. μ Ts were bioprinted into 1% XG-MA support bath and

encapsulated into 1% gelatin and 0.05% LAP (w/v) supplemented with growth factors as follows: TGF- β 3 for 4 weeks, CTGF+TGF- β 3 for 4 weeks, or no growth factors in the ink but added in the medium (CDM+ group). A) Histological assessment of chondrogenic markers: H&E, Alcian Blue (sGAG), and Alizarin Red (calcium deposits). Scale bar:120 μ m. B–G) Biochemical quantification: DNA (B), sGAG (C), total collagen (D), sGAG/DNA (E), collagen/DNA (F), and sGAG/collagen (G). Outliers were identified using a ROUT test. Data were analysed by one-way ANOVA followed by Tukey's post hoc test. Statistical significance was defined as $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

DNA content was comparable across groups (Fig. 7.5 B). By the end of the culture period, Alcian blue staining revealed robust sGAG deposition in all groups, though slightly reduced in the CTGF+TGF- β 3 (Fig. 7.5 A, C). Biochemical quantification confirmed that sGAG levels were significantly higher in CDM+, TGF- β 3 groups compared to CTGF+TGF- β 3, while no significant differences were found between the soluble TGF- β 3 group and constructs with laponite-incorporated TGF- β 3, indicating successful loading and release of the factor (Fig. 7.5 C). These differences were also reflected when sGAG content was normalised to DNA (Fig. 7.5 E). Collagen synthesis was lowest in the CTGF+TGF- β 3 group (Fig. 7.5 D, F).

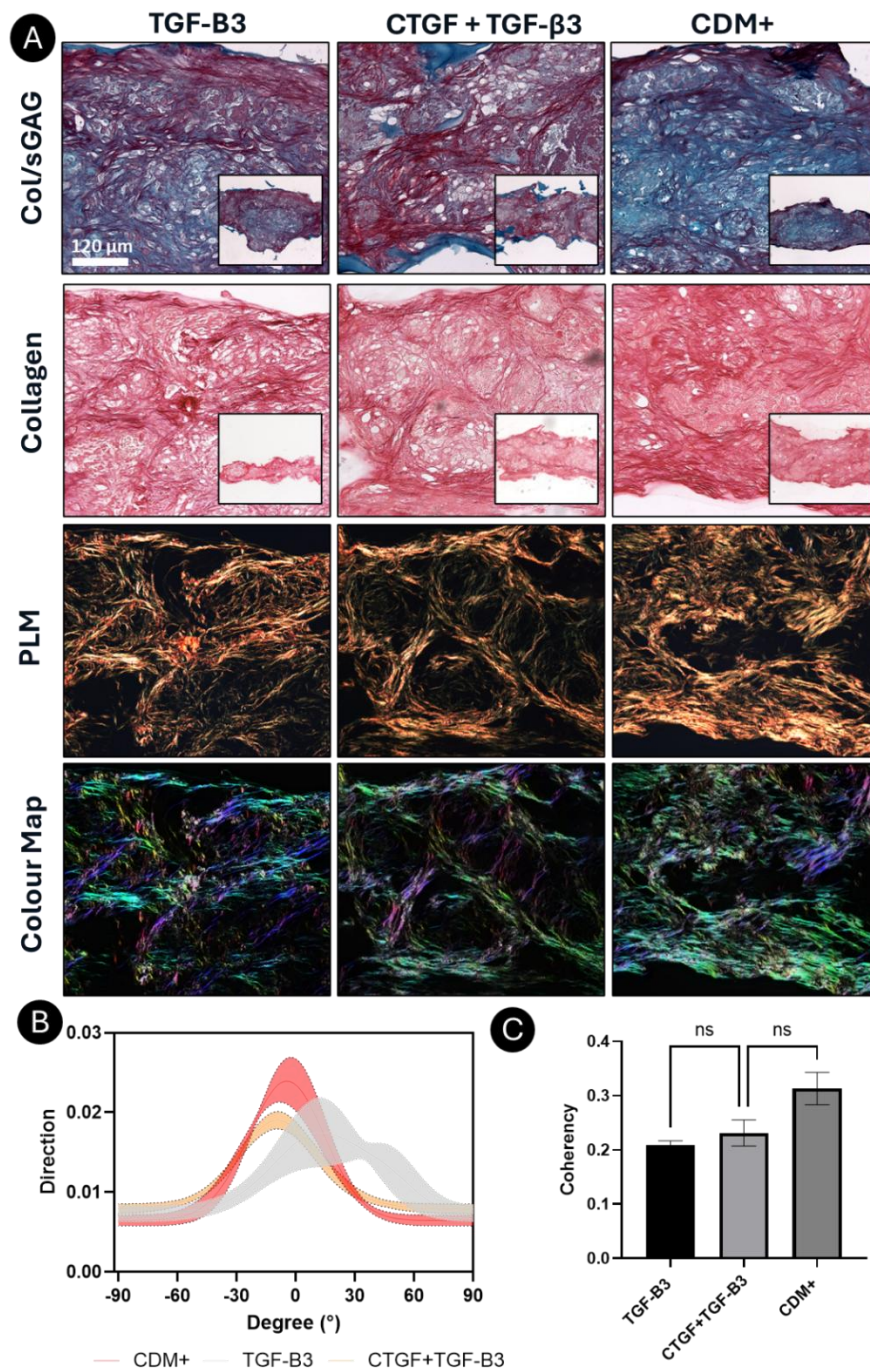


Figure 7.6. Printed μ Ts into laponite based bioinks exhibit distinct collagen alignment profiles under distinct growth factor regimes. A) Histological staining with Picrosirius Red/Alcian Blue to visualise both collagen and sGAG deposition over 28 days, alongside polarised light microscopy (PLM) and OrientationJ-derived colour maps. Colour hue indicates fibre orientation: blue/cyan = 0°, pink/red = 90°. Scale bar = 120 μ m. B) Average collagen fibre orientation under different growth factor regimes with laponite-mediated slow release. C) Fibre coherency, where values close to 1 represent highly aligned fibres and values near 0 represent dispersed orientations (n = 3). Scale bar = 200 μ m. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc test. Statistical significance was set at $p < 0.05$.

This was further confirmed by Picrosirius red staining (Fig. 7.6 A). Notably, collagen deposition appeared robust in all groups, and polarised light imaging revealed preferential collagen alignment across all constructs (Fig. 7.6 A). No major differences in collagen fibre alignment and coherency were detected between the CDM+, CTGF+TGF- β 3, and TGF- β 3 groups (Fig. 7.6 B, C). These findings demonstrate the potential of laponite-mediated growth factor delivery to drive the organisation and composition of engineered region-specific meniscal tissues.

7.3.5 Growth factor regime and support bath confinement promote a fibrocartilage-like phenotype in printed μ Ts

Having shown that loading CTGF and TGF- β 3 into the bioink can drive fibrocartilaginous differentiation of bioprinted μ Ts within the support bath, we next characterised the tissue phenotypes supported by these bioink combinations. To this end, qPCR analysis and immunohistology staining for collagen deposition were performed after 4 weeks of *in vitro* culture to assess gene expression profiles associated with fibrocartilaginous differentiation (Fig. 7.7).

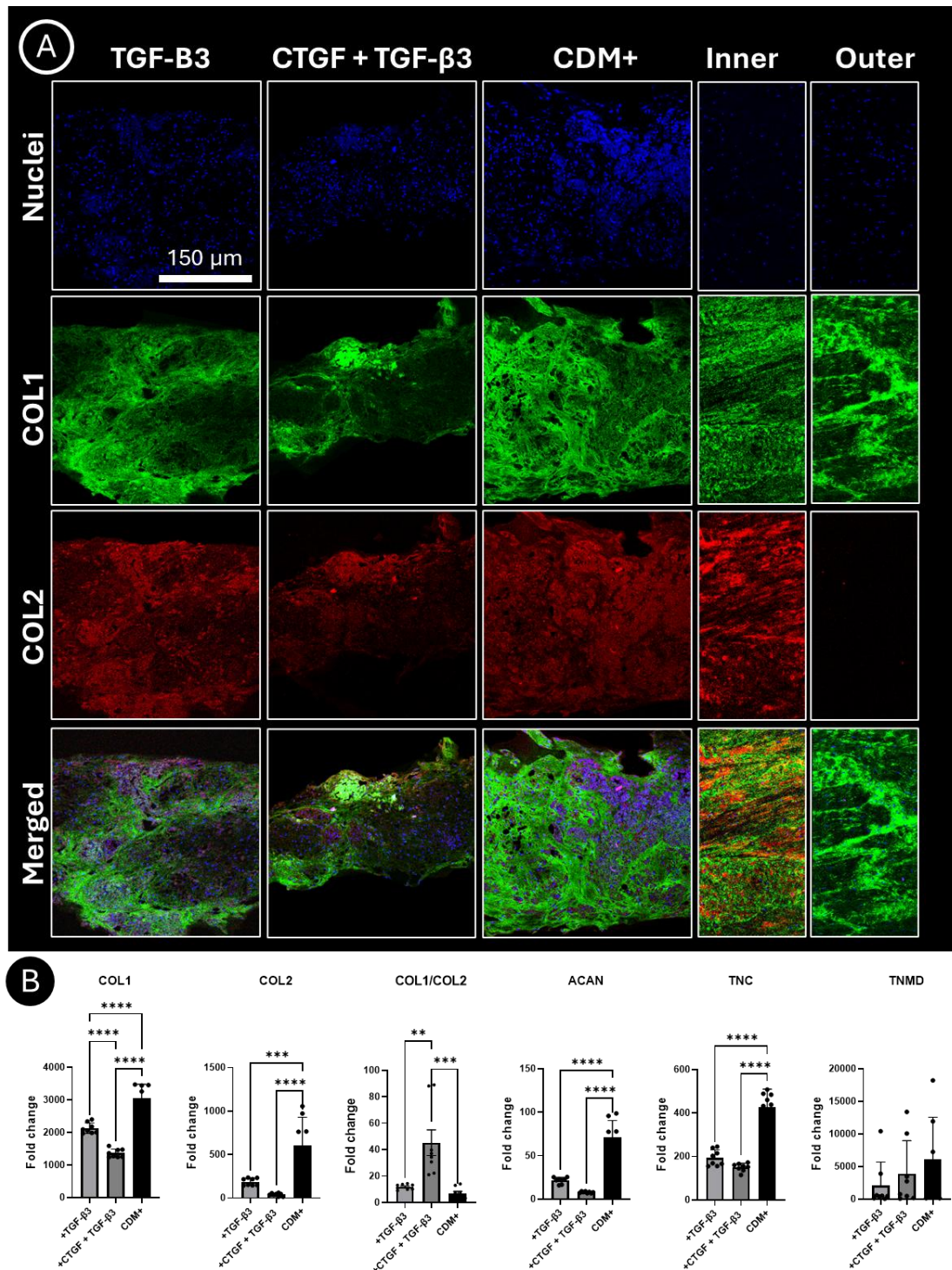


Figure. 7.7 Localised growth factor delivery alters collagen deposition and gene expression in printed μ Ts. A) Immunohistology staining for collagen I (green) and collagen II (red) in bioprinted μ Ts. Native inner and outer meniscus regions are shown for comparison, highlighting regional differences in collagen deposition. Scale bar: 150 μ m. B) qPCR analysis of bioprinted μ Ts with localised growth factor delivery, showing differential expression of chondrogenic and fibrochondrogenic markers, including collagen I (COL1), collagen II (COL2), COL1/COL2 ratio, aggrecan (ACAN), tenascin (TNC), and tenomodulin (TNMD). Data were analysed using one-way ANOVA, with significance accepted at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Distinct differences in collagen staining were observed between groups (Fig. 7.7). μ Ts cultured with printed TGF- β 3 showed strong deposition of both collagen types I and II, consistent with the inner meniscus phenotype (Fig. 7.7 A). In contrast, CTGF+TGF- β 3 promoted robust collagen type I but weaker collagen type II deposition, more closely resembling the outer meniscus (Fig. 7.7 A). Interestingly, CDM+ and TGF- β 3 groups displayed comparable staining patterns, both with high levels of collagens I and II (Fig. 7.7 A). Gene expression profiles also reflected these trends. Collagen type I and II expression was highest in the CDM+ group, where TGF- β 3 was added directly to the media (Fig. 7.7 B). Collagen type II expression did not differ significantly between the two groups with printed growth factor delivery, but analysis of the COL1/COL2 ratio revealed a marked distinction: CDM+ and printed TGF- β 3 groups showed lower ratios, while CTGF+TGF- β 3 exhibited the highest ratio, indicating that CTGF preferentially supports collagen type I over collagen type II expression (Fig. 7.7 B). Aggrecan (ACAN) expression was also elevated in the printed TGF- β 3 group compared to the printed CTGF+TGF- β 3 group, consistent with its role in promoting a more hyaline cartilage-like phenotype (Fig. 7.7 B).

7.3.6 Dual-cartridge bioprinting enables precise fabrication of scaled-up regionally defined meniscal grafts

Having demonstrated that controlled release of CTGF + TGF- β 3 alongside printed μ Ts supports fibrocartilaginous differentiation, while release of TGF- β 3 alone supports hyaline-like differentiation, this thesis next explored bioprinting a zonally defined meniscal graft. Specifically, several circumferential lines of μ Ts encapsulated in a laponite/gelatin bioink loaded with CTGF + TGF- β 3 were bioprinted for the outer region of the meniscal graft (Fig. 7.8).

A Dual Cartridge 3D Bioprinting of Regionally Defined Meniscal Grafts

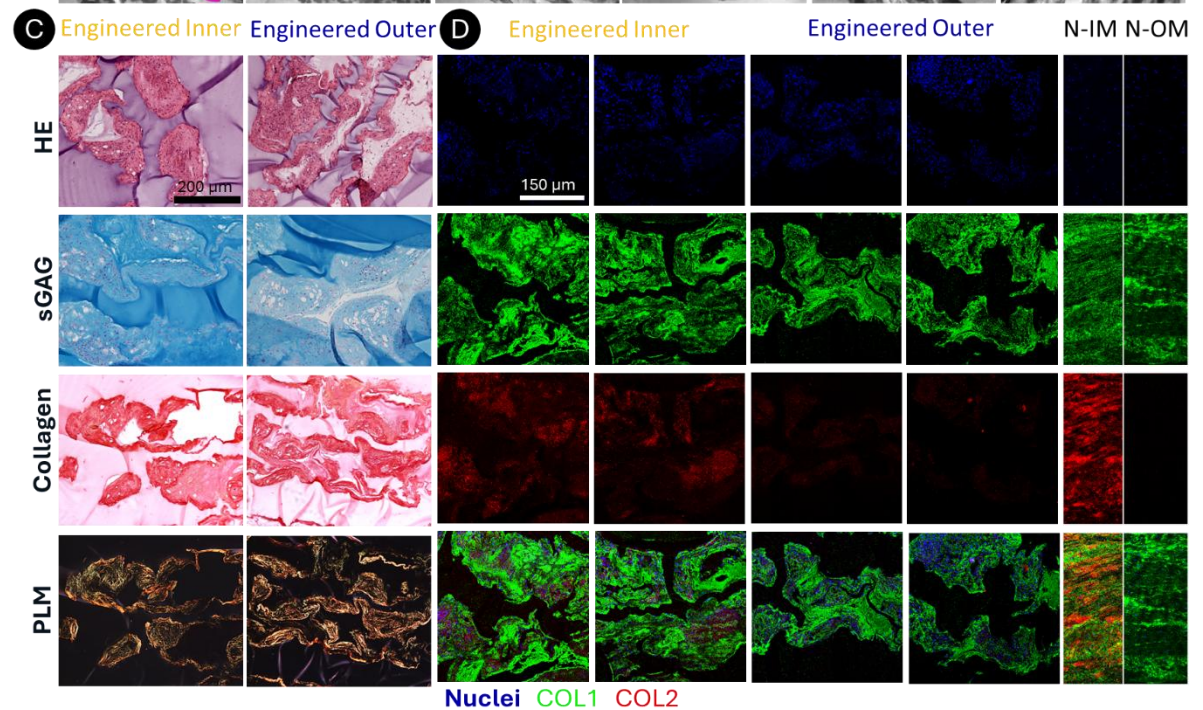
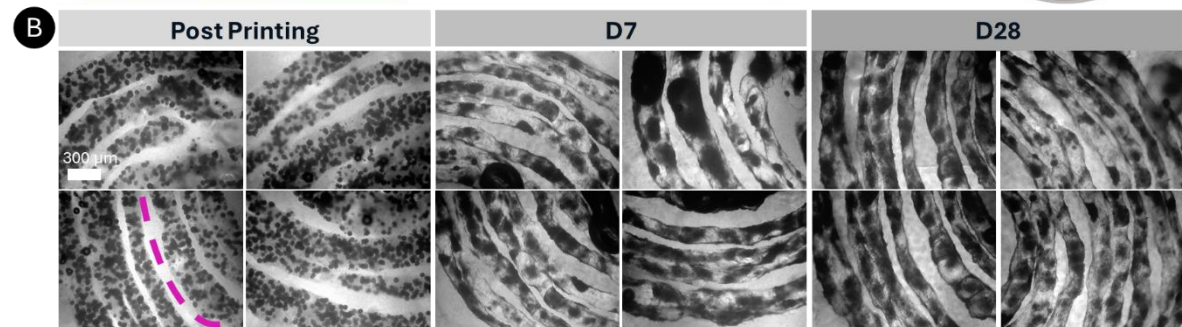
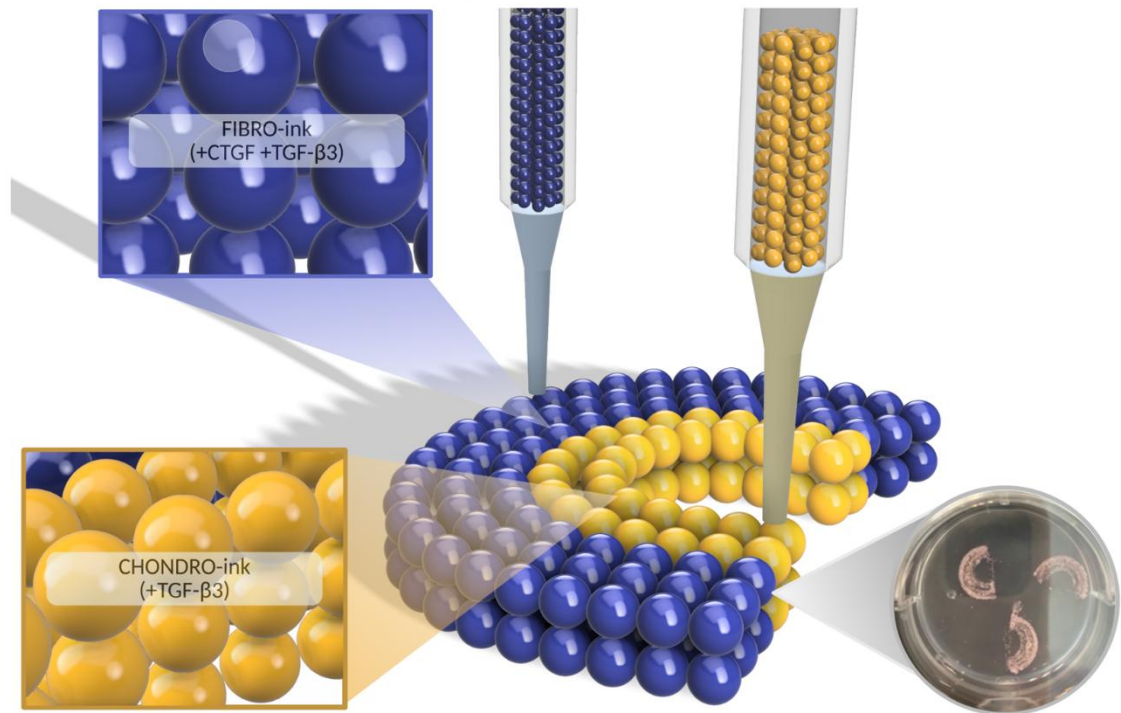


Figure 7.8. Dual-cartridge bioprinting enables precise fabrication of scaled-up grafts and region-specific constructs for meniscal regeneration. (A) Schematic of the dual-cartridge bioprinting workflow: MSC-derived μ Ts are deposited within an XG-MA support bath using two bioinks, a chondro-ink (+TGF- β 3) and a fibro-ink (+CTGF + TGF- β 3), to recapitulate meniscal zonal composition. Zonal patterning strategy: the outer zone is printed with fibro-ink (CTGF + TGF- β 3) and the inner zone with chondro-ink (TGF- β 3). Macroscopic image of three zonally defined bioprinted menisci immediately post-printing (day 0) in a 6-well plate. (B) Brightfield microscopy images of bioprinted filaments in the engineered inner and outer zones at day 0 and day 28; the dashed line marks the boundary between zones. Scale bar: 300 μ m. (C) Histological evaluation of engineered inner and outer zones: H&E, Alcian Blue (sGAG), Picrosirius Red (collagen) and PLM. Scale bar: 200 μ m. (D) Immunofluorescence for collagen I, collagen II, with DAPI for nuclei; native inner and outer meniscus shown for comparison. Scale bar: 150 μ m.

For the inner region, additional circumferential lines were printed inside the outer ring, using μ Ts encapsulated in a laponite/gelatin bioink loaded with TGF- β 3 only (Fig. 7.8 A). This dual-bioink approach required the use of two separate printheads, with printhead 1 depositing the outer region and printhead 2 depositing the inner region (Fig. 7.8 A-G). Although the two-printhead process is more complex than single-printhead printing, the process was optimised considering the time sensitivity of handling μ Ts. Filaments were spaced 50–100 μ m apart to prevent unwanted fusion (preserving filament anisotropy) while still producing a more compact tissue compared to earlier constructs. Macroscopically, the tissue maintained a meniscus-like shape (Fig. 7.8 D). Microscopically, the filaments appeared well defined and closely packed, and by day 7, the μ Ts had already begun fusing, forming a more continuous tissue. By day 28, the gaps between filaments were further filled, indicating sustained viability and ECM deposition (Fig. 7.8 D). Histological staining was then performed, and the engineered construct was divided into outer and inner regions (Fig. 7.8 E). The inner zone stained more intensely for sGAG compared to the outer zone, consistent with the effect of TGF- β 3 in promoting GAG deposition, even in a scaled-up construct with the same basal media (chemically defined media without TGF- β 3 and/or CTGF). This confirmed localised delivery and rapid local uptake of the growth factor. Collagen staining revealed no overall differences in amount or in fibre alignment, in line with our previous findings. However, collagen type-specific

staining showed that the outer zone was more positive for collagen type I and less for type II, whereas the inner zone contained both collagen types I and II (Fig. 7.8 D).

Together, these results demonstrate the successful bioprinting of a biomimetic and zonally defined meniscal graft using MSC-derived μ Ts with spatially defined co-delivery of growth factors within a scaled-up construct.

7.4 Discussion

This study demonstrates the feasibility of engineering zonally defined meniscal grafts by combining MSC-derived μ Ts with spatially patterned growth factor delivery through dual-cartridge bioprinting. Unlike previous scaffold-based or scaffold-free strategies that either lacked zonal fidelity or relied on meniscus-derived cells [44, 427, 428], this approach integrates a scalable, clinically accessible cell source with a dual-bioink system capable of recapitulating regional heterogeneity. To capture this heterogeneity, we expanded beyond TGF- β 3 alone and incorporated CTGF to create controlled growth factor gradients in printed constructs. Previous studies had shown that CTGF enhances collagen type I deposition while suppressing collagen type II, whereas TGF- β 3 promotes both sGAG and collagen type II accumulation [154, 155, 440]. Based on this rationale, we first evaluated different growth factor regimes in self-assembled μ T constructs to determine their suitability for zonal printing.

μ Ts were fused in agarose moulds under five conditions: TGF- β 3 alone, CTGF alone, sequential CTGF $\rightarrow$ TGF- β 3, sequential TGF- β 3 $\rightarrow$ CTGF, and CTGF + TGF- β 3. This 3D μ T system allowed us to directly assess phenotypical responses, which differ from those reported in 2D cultures or cell-laden hydrogels [155]. The results demonstrated that CTGF alone was insufficient for promoting fibrochondrogenic differentiation, as evident by relatively low levels of sGAG and collagen deposition. However, when combined with TGF- β 3, particularly during the early induction phase, CTGF modulated matrix composition to more closely reflect native meniscal tissue, which contains 1–3% sGAG by wet weight compared to 5–7% in hyaline cartilage [441]. Constructs treated with CTGF + TGF- β 3 displayed reduced sGAG but improved collagen production, more representative of the fibrocartilaginous phenotype observed in the outer zone of the meniscus. Immunostaining further confirmed that TGF- β 3 promoted strong collagen type I and II expression, while CTGF shifted the balance toward higher collagen I and markedly reduced collagen II deposition. These observations provided a clear rationale for selecting TGF- β 3 alone to represent the inner zone and CTGF + TGF- β 3 for the outer zone in subsequent bioprinting studies.

By leveraging the self-assembly potential of μ Ts [237, 337] and the controlled release properties of laponite-based bioinks [138], we were able to bioprint zonally defined

meniscal constructs that reproduced key features of the native tissue. All printed groups showed high viability. Constructs cultured with CTGF + TGF- β 3 exhibited fibrocartilaginous characteristics consistent with the outer meniscus, whereas TGF- β 3 alone induced a more hyaline-like phenotype consistent with the inner zone. The major novelty of this work lies in the successful use of dual-cartridge μ T bioprinting to achieve zonal μ T and growth factor patterning within a single meniscal graft. Previous studies employing growth factor-loaded microparticles [356, 442, 443], homogeneous bioinks [444, 445], or meniscus progenitor cells [44, 446-448] achieved only partial zonal organisation or were constrained to smaller scales. Here, the integration of two distinct bioinks enabled precise spatial patterning of μ T and growth factor combinations supportive of either inner or outer zone phenotypes, while maintaining filament fidelity and tissue compactness at a macroscale. From a translational perspective, the use of laponite as a component of the bioink needs careful consideration. While laponite has been widely explored in TE due to its favourable rheological properties and ability to bind and release growth factors [138, 228], it is not currently approved as an implantable material for clinical use. Therefore, prior to clinical translation, its safety, degradation profile, and long-term biological interactions would need to be thoroughly evaluated in vivo and assessed through appropriate regulatory pathways. Strategies such as reducing laponite content, ensuring its clearance over time, or replacing it with clinically approved alternatives may be required. However, its use in this study provides a valuable proof-of-concept for controlled growth factor delivery within bioprinted μ T-based constructs, which can inform the development of more clinically translatable systems.

Histological analysis of scaled-up constructs confirmed effective spatial delivery of growth factors: the inner zone displayed higher sGAG accumulation together with strong collagen II and collagen I deposition, while the outer zone exhibited lower sGAG levels, negligible collagen II, and robust collagen I deposition. The introduction of laponite within the bioink, influenced the fusion behaviour of the μ Ts. While laponite enables controlled and localised presentation of bioactive factors, its presence may also act as a physical barrier or alter the microenvironment, potentially hindering or slowing complete μ T fusion. Collagen organisation appears less homogeneous and less aligned compared to constructs presented in previous chapters. This highlights a key limitation of the current approach, where optimisation of bioink composition is required to balance growth factor delivery with

efficient tissue integration. Future work should focus on refining laponite concentration and investigating release kinetics to identify the minimum effective concentration required to achieve sustained growth factor delivery while minimising interference with μ T fusion.

These results demonstrate that laponite-based growth factor delivery can successfully promote region-specific differentiation in large, engineered constructs—a persistent challenge in zonal tissue engineering. Compared with our earlier μ T fusion experiments, this study marks a significant step forward by translating small-scale differentiation findings into large, architecturally defined grafts that preserve biomimicry during *in vitro* culture. Although collagen deposition improved with combined growth factor regimes, total collagen content remained below native levels, and collagen fibre anisotropy has not yet been fully recapitulated. Future work should incorporate mechanical stimulation and extended culture periods to enhance collagen maturation and alignment, as well as evaluate the mechanical properties of the grafts under physiologically relevant loading. Ultimately, *in vivo* validation will be required to assess integration and long-term functionality.

In summary, this work introduces a novel strategy for the biofabrication of zonally defined meniscal grafts by combining MSC-derived μ Ts with dual-bioink delivery of growth factors. By uniting scalable cell sources, precise spatial patterning in physically constraining support baths and sustained but localised growth factor presentation, this approach advances beyond prior scaffold-based or single-bioink methods and establishes a robust platform for developing clinically relevant meniscal replacements.

7.5 Conclusion

This study establishes a framework for engineering zonally defined meniscal grafts by integrating MSC-derived μ Ts with dual-cartridge bioprinting and laponite-based growth factor delivery. The constructs successfully recreated key biochemical gradients of the native tissue: the inner zone displayed higher sGAG (~1–3% of wet weight in native meniscus) and collagen II deposition, while the outer zone exhibited lower sGAG content and predominantly collagen I, consistent with its fibrocartilaginous character. Importantly, these zonal differences were preserved in scaled-up grafts despite shared media conditions, underscoring the effectiveness of localised growth factor presentation. While total collagen

deposition and fibre anisotropy remain below native benchmarks, this approach advances beyond previous scaffold-based or single-bioink strategies by combining scalable cell sources, localised biochemical and biophysical guidance, and spatial fidelity in a single graft. Taken together, these findings represent a significant step toward the development of clinically relevant, biomimetic meniscal replacements.

Chapter VIII: Discussion

The overarching objective of this thesis was to develop and apply a versatile μ T-based bioprinting platform using clinically relevant cell sources from human and caprine bone marrow to engineer biomimetic, functional musculoskeletal tissues. Emphasis was placed on ECM synthesis, collagen organisation, region-specific collagen deposition, and the mechanical anisotropy of the resulting constructs. Across all studies, microtissue fusion was treated as a central readout of system performance, as it represents the key determinant of construct cohesion and is therefore essential for generating mechanically robust, clinically relevant grafts. This thesis established a biofabrication strategy that coupled cellular self-organisation with 3D and 4D embedded bioprinting to generate anisotropic musculoskeletal grafts. First, it was demonstrated that early-stage μ T fusion enhanced chondrogenesis and enabled scaling to tissue-relevant dimensions. This fusion capacity depended strongly on the establishment of cell–cell and cell–ECM interactions during μ T fusion, and it was further dictated by the maturation state of the μ Ts before fusion. Second, increasing oxygen tension during the culture of scaled-up, clinically relevant cartilage grafts supported the development of a more biomimetic cartilaginous tissue. These MCSs derived μ Ts, harvested at an early maturation stage (F-D2), were then employed as building blocks in the development of a new bioprinting strategy. This approach formed the basis for the development and refinement of a bioprinting platform that can be adapted to other musculoskeletal tissues by tuning the physical properties of the microenvironment, thereby mimicking aspects of tissue self-organisation in line with developmental engineering paradigms. Finally, the platform was used with a specific focus on meniscal TE, aiming to recreate its inherent heterogeneity and intricate architecture by controlling the delivery of distinct growth factors and by systematically modifying both the bioink and the support bath. Together, these advances defined a reproducible framework for bioprinting large, anisotropic, functionally specialised musculoskeletal tissues by spatially pattern μ Ts.

A central finding in Chapter III is that the maturation state of microtissues before fusion governed both fusion kinetics and regulated chondrogenesis. While μ T fusion has been widely studied [161, 170-173, 237, 273, 336], the impact of maturation on subsequent cartilage formation has been less explored. Early-stage μ Ts (F-D2) fused more rapidly and uniformly and exhibited higher sGAG and collagen accumulation. This behaviour

corresponded with elevated early N-cadherin expression, consistent with stronger cell–cell adhesion, and with increased vimentin expression during later fusion and matrix maturation [101, 283, 285, 286, 376]. Importantly, increasing μ T number to enlarge constructs did not impede fusion, supporting the feasibility of generating scaled-up grafts from human MSC μ Ts. As constructs were scaled up (from 0.2mm to 5mm in diameter), collagen deposition diminished in the core at the highest densities and dimensions, suggesting that nutrient transport is a challenge in these cell-dense tissues [291]. Adjusting oxygen tension partially mitigated these central limitations without inducing hypertrophy in the presence of TGF- β 3. These observations indicated that, despite robust fusion, engineered control of mass transport during maturation (e.g., perfusion bioreactors, transient porogens, or bioink additives) and of fusion geometry/direction will be required to further enhance collagen content and organisation toward native levels.

To address tissue organisational control, Chapter IV developed an embedded bioprinting platform for high-density μ Ts to produce spatially organised musculoskeletal constructs. This chapter investigated how sacrificial bioinks, nozzle geometry, μ T density, and support-bath mechanics modulated μ T fusion and tissue maturation. Although μ T printing has been reported, prior studies often lacked organisation and showed poor μ T fusion, limiting function [65, 173, 212]. At the beginning of this thesis, no standardised method had been established to reliably print μ Ts at high throughput, promoting fusion and driving tissue maturation. This study investigated the critical parameters influencing successful extrusion-based bioprinting and subsequent maturation of μ T-laden constructs for cartilage TE. Specifically, two fully sacrificial inks, gelatin and methylcellulose, were evaluated based on the logic that rapidly degrading hydrogels would allow μ T fusion, improve tissue cohesiveness and therefore chondrogenesis compared to more persistent hydrogels [66, 218]. Gelatin outperformed methylcellulose by balancing shear protection during extrusion with rapid post-print clearance, enabling efficient μ T fusion. An optimal density of $\sim 45,000$ μ T mL⁻¹ (22G nozzle, ~ 175 μ m spheroids) produced continuous filaments; lower densities yielded gaps, whereas higher densities caused clogging and disrupted filament continuity. Beyond the ink, the support bath proved critical: unlike conventional sacrificial baths removed before culture [204, 211, 314], a non-sacrificial, crosslinkable bath was needed to impose stable physical boundaries. Unmodified XG supported only short-term

confinement, whereas XG-MA preserved tissue geometry during culture, limited tissue shrinkage, and enabled sustained μ T fusion and ECM organisation. Both XG and XG-MA were shear-thinning and thixotropic, but the crosslinked stability of XG-MA maintained spatial confinement and modulated collagen organisation.

The support bath formulations developed in Chapter V functioned as a temporally adapting microenvironment that applied radial confinement and altered the printed filament diameter over time. Analogous to DE paradigms in which developing tissues experience mechanical confinement from surrounding structures [81], MSC differentiation is sensitive to geometric cues and matrix stiffness [296, 300, 341, 374, 375]. In this chapter, it was shown that tuning bath stiffness modulated YAP activity in μ Ts and directed MSC differentiation. Softer baths favoured chondrogenic gene expression and collagen II/sGAG deposition; intermediate baths supported fibrochondrogenic features; and stiffer baths promoted fibrous/myofibroblastic phenotypes with increased type I collagen and α -SMA expression. Polarised light imaging confirmed that these boundary conditions also guided collagen alignment along the print path, yielding anisotropic architectures reminiscent of all the musculoskeletal tissues [4, 70, 362]. These findings demonstrate that altering the physical properties of the support bath can be used to tune the phenotype of printed MSC μ Ts. By leveraging the distinct mechanical properties of different baths, specific printing architectures were designed to mimic collagen organisation in three target tissues: an arcade-like pattern for articular cartilage [110], circumferential trajectories for the meniscus [421], and straight paths for ligament [70], thereby further enhancing tissue anisotropy. In this setting, printed μ Ts aligned along the imposed 3D printing paths, and their fusion and remodelling were directed by the mechanical constraints imposed by the bath, simultaneously influencing collagen anisotropy and the phenotypic response via mechanotransductive cues. Collectively, this 4D support-bath design links controlled mechano-stimulation to control of ECM orientation and lineage specification in scaled μ T-based constructs.

Permanent confinement imposed by the support bath, however, can limit lateral fusion between adjacent filaments in larger, more complex printed grafts. Chapter VI addressed this by blending XG-MA with unmodified XG to form a semi-interpenetrating (semi-IPN) network, introducing controllable degradability while preserving shear-thinning behaviour,

print fidelity, and chondrogenic performance. Given the sacrificial nature of XG, it was hypothesised that adding it to XG-MA would maintain the physical properties of the bath ideal for μ T printing and post-printing maturation, while accelerating long-term bath clearance during *in vitro* culture. Across a broad ratio range, rheology remained comparable, indicating that differences in compaction and fusion primarily reflected degradation dynamics rather than altered printing mechanics. Increasing XG content elevated swelling and relaxation, consistent with reduced effective crosslink density and a more degradable network, facilitating post-print filament fusion without compromising viability or inducing hypertrophy/mineralisation. Excessive degradability (3:1 XG: XG-MA), however, weakened boundary conditions and reduced collagen alignment. A 1:1 blend achieved a favourable balance, maintaining anisotropy while enabling progressive bath clearance and improved mechanical integration between filaments. This versatile, partially sacrificial bath reconciled the competing demands of geometric stability during printing and scaffold removal during maturation, advancing the fabrication of large, anisotropic, scaffold-free musculoskeletal grafts. Future work should assess the *in vivo* compatibility of degradation products and explore longer degradation profiles.

Finally, Chapter VII leveraged the platform's precision to fabricate zonally defined meniscal grafts using MSC-derived μ Ts and laponite-based delivery of TGF- β 3 and CTGF. Although these growth factor regimes have previously generated fibrocartilaginous constructs in 2D and 3D cultures with MSCs and meniscal cells [154, 155, 431], they have not been integrated within a μ T bioprinting framework capable of simultaneously controlling architecture, anisotropy, and local growth factor-controlled release. Incorporation of laponite into the printing system enabled spatially resolved, sustained presentation of morphogens within printed filaments, providing a route to pattern region-specific phenotypes within a single construct. Different growth factor exposure in fused μ Ts confirmed that CTGF alone suppressed cartilage-like matrix assembly, whereas CTGF + TGF- β 3 moderated sGAG content and enhanced collagen type I deposition, approximating the outer meniscus fibrocartilaginous profile. In contrast, TGF- β 3 alone supported an inner-zone, hyaline-like phenotype with a greater cartilaginous matrix phenotype. Dual-cartridge embedded bioprinting was then used to spatially pattern these distinct cue combinations within one construct at clinically relevant scales, while preserving cell viability, μ T fusion,

and establishing anisotropy. Laponite-mediated retention of growth factors within filaments supported sustained intra-filament release, enabling stable, spatially distinct regional phenotypes over the culture period [138]. Conceptually, this extended dual-cartridge printing strategies by coupling localised, differential growth factor exposure to a μ T-based bioprinting platform. In doing so, the work demonstrated that local growth factor delivery can be coupled with the bioprinting of anisotropic tissues to generate meniscal grafts with both structural and compositional organisation that more closely recapitulate the native tissue, thereby enhancing the translational potential of μ T-based meniscus engineering.

A practical strength of this platform is its reliance on scalable MSC-derived μ Ts and on process inputs that can be controlled and standardised. Rather than depending on complex, hard-to-synthesise, extracted biomaterials or highly specialised hardware, the system is built around parameters that can be easily measured and then tuned. Key inputs include the maturation state of the μ Ts, the viscosity of the carrier ink, the dimensions of the printing nozzle, and the stiffness and/or degradability of the support bath. Each of these can be defined within specific operational ranges, making them suitable for the development of standard operating procedures (SOPs) and quality-control thresholds, which are essential for regulatory progression and eventual clinical translation. The ability to perform high-density printing while preserving cell viability and supporting sustained μ T fusion is particularly important for generating patient-sized grafts, where large tissue volumes are required without compromising function. Moreover, the same core platform can also be tuned by adjusting these physical and biochemical inputs to guide MSC μ Ts toward different lineage outcomes, enabling the fabrication of a range of musculoskeletal tissues (e.g., cartilage, fibrocartilage, or ligament-like constructs) using a common, adaptable manufacturing framework.

However, while this bioprinting strategy clearly enabled the biofabrication of musculoskeletal tissues that mimic key features of the native tissues, there are still limitations that need to be addressed.

- I. Cell source: Most experimental work relied on caprine cells (with the exception of Chapter III). Although the goat is a widely accepted large-animal preclinical model in musculoskeletal tissue engineering, rigorous validation with human-derived

MSCs and μ Ts is still required. Systematic studies across multiple donors, ages, and disease backgrounds will be essential to confirm that the observed fusion behaviour, differentiation profiles, and architectural outcomes translate robustly to human cells.

- II. Cell and μ T manufacturing at scale: μ T bioprinting inherently demands very high cell numbers, especially when targeting patient-sized grafts. To move toward translation, a high-throughput, closed bioreactor workflow is needed to expand MSCs without altering their genetic or epigenetic profiles, and to mass-produce μ Ts in a controlled and reproducible manner. Streamlined, automated aggregation and differentiation protocols that minimise manual handling would also reduce contamination risk and variability during expansion, μ T formation, and maturation.
- III. Mechanical performance gap: The engineered, scaffold-free constructs generated here do not yet fully match the mechanical properties of native musculoskeletal tissues. Closing this gap will likely require coordinated strategies to further enhance collagen maturation and crosslinking, to apply more targeted and physiologically relevant mechanostimulation (e.g., multiaxial loading regimes), and to improve nutrient and factor transport during maturation so that matrix deposition and organisation can progress toward native levels throughout the entire construct.
- IV. Inter-filament fusion post-printing: Even though a more degradable support bath was introduced and substantially improved fusion within individual filaments, complete fusion between adjacent filaments and layers in scaled, highly anisotropic grafts remains incomplete. This incomplete inter-filament integration limits overall mechanical competence and may introduce weak interfaces within the construct. Future work will need to optimise parameters such as filament spacing, bath degradation kinetics, fusion time windows, and potentially incorporate additional cues that promote inter-filament bridging in order to achieve fully cohesive, bulk tissues.
- V. Pre-clinical translation and graft source: From a translational perspective, autologous implantation would be ideal, as non-autologous grafts carry a risk of immune reactions that could impair integration and healing. However, establishing truly autologous workflows in a caprine model is logistically demanding, time-consuming, and costly, particularly when very high cell numbers and complex

culture timelines are required. Strategies to either streamline autologous MSC/ μ T production or to develop safe, immune-compatible allogeneic approaches will be necessary before this platform can be realistically positioned for clinical application.

This thesis advanced μ T-based bioprinting from a promising but relatively unexplored concept to a versatile, mechanobiology-oriented additive manufacturing strategy for the engineering of anisotropic musculoskeletal grafts. A central theme throughout the work was that microtissue fusion was not only a structural requirement but also a key driver of differentiation, ECM organisation, and ultimately mechanical function. Early fusion of less mature μ Ts was shown to establish strong chondrogenic trajectories, with enhanced ECM deposition and more homogeneous tissue formation compared to more mature μ Ts. Building on this, embedded bioprinting into support baths with programmable stiffness and time-dependent behaviour translated these biological trajectories into aligned architectures that are relevant for load-bearing tissues, by constraining the printed μ Ts and therefore, guiding collagen alignment along the print path. The introduction of semi-interpenetrating network (semi-IPN) support baths further reconciled two often competing demands: maintaining high geometric fidelity after printing while still permitting sufficient degradation and relaxation to allow inter-filament fusion and the development of cohesive, anisotropic tissues at larger scales. Finally, spatial patterning of growth factors using dual bioinks and laponite-based delivery enabled regional control over cell phenotype, exemplified by the generation of zonally defined meniscal grafts that recapitulate inner (more cartilaginous) and outer (more fibrous) tissue characteristics within a single construct.

Together, these elements defined an integrated framework in which cell source, μ T maturation, mechanical confinement, matrix organisation, and zonal growth factor delivery were deliberately coordinated. As a result, the platform supports the fabrication of large, structurally organised, and functionally specialised musculoskeletal grafts that move μ T-based bioprinting closer to realistic translational implementation.

8.1 Future Work and Translational Outlook

While this bioprinting strategy enabled the fabrication of musculoskeletal tissues that recapitulate key structural and biological features of native tissues, several challenges remain to be addressed to advance this platform toward clinical application in future work.

I. Advanced mechanical stimulation strategies. The constructs generated in this work were primarily matured under static or limited dynamic culture conditions. As previously described, native musculoskeletal tissues are exposed to complex mechanical environments, including compression, tension, and shear. Future studies should incorporate more physiologically relevant, multiaxial loading regimes using advanced bioreactor systems to promote collagen alignment, matrix maturation, and improved mechanical performance. The integration of controlled mechanical cues during culture may be critical to achieving tissue properties closer to native levels.

II. Nutrient transport and core tissue maturation. As construct size increases, limitations in nutrient and oxygen diffusion become more pronounced, potentially leading to heterogeneous tissue development and the formation of collagen-poor or underdeveloped cores. The use of perfusion bioreactors or dynamic culture systems that enhance mass transport may help overcome these limitations. In addition, optimisation of construct architecture, porosity, and filament spacing could further support uniform tissue maturation throughout the graft.

III. Translation from caprine to human cell sources. Most experimental work in this thesis relied on caprine MSCs, which represent a well-established large-animal model. However, translation to human application will require systematic validation using human-derived MSCs and μ Ts. Donor variability, age, and disease state of the patients are likely to influence cell behaviour, fusion capacity, and differentiation potential. Therefore, future studies should include multi-donor human cell populations to assess the reproducibility and robustness of the platform.

IV. Bioink and material optimisation for clinical use. While materials such as laponite and XG-based support baths provide important functionality for printing and growth factor delivery, their translational relevance requires further consideration. Future work should focus on optimising material composition to minimise potential interference with μ T fusion,

ensure controlled degradation, and evaluate long-term biocompatibility and clearance. In particular, systematic studies on laponite concentration and growth factor release kinetics will be necessary to identify the minimum effective dose that maintains biological efficacy while improving tissue integration.

From a regulatory perspective, any material intended for clinical application must comply with Good Manufacturing Practice (GMP) requirements, which define strict standards for manufacturing processes, quality control, reproducibility, and safety. In addition, biomaterials must undergo extensive biocompatibility testing in accordance with ISO 10993 guidelines to ensure they do not elicit adverse biological responses *in vivo*.

In contrast, several naturally derived biomaterials such as gelatin and collagen have already demonstrated strong clinical relevance due to their inherent biocompatibility and established regulatory history and are widely used in approved medical devices and drug formulations. By comparison, materials such as laponite, while highly promising for controlled growth factor delivery, are not currently approved as implantable biomaterials for clinical use. Therefore, prior to translation, their safety, degradation profile, long-term biodistribution, and clearance must be rigorously evaluated under GMP-compliant conditions. Future work should focus on optimising material composition to minimise potential interference with μ T fusion, while also exploring strategies to reduce laponite content or replace it with clinically validated alternatives. In particular, systematic studies on laponite concentration and growth factor release kinetics will be necessary to identify the minimum effective dose that maintains biological efficacy while improving tissue integration and facilitating regulatory approval.

V. Preclinical validation and clinical translation. Ultimately, *in vivo* validation will be essential to assess graft integration, mechanical functionality, and long-term stability. This includes evaluating the performance of bioprinted constructs in relevant animal models under physiological loading conditions. rigorous preclinical validation will be required in relevant large-animal models, such as ovine or caprine meniscal defect models, which better recapitulate joint size, loading conditions, and healing responses observed in humans. Future studies should assess not only graft integration and retention within the defect site, but also mechanical functionality under physiological loading, long-term stability, and resistance to degeneration. Key outcome measures should include histological integration

with native tissue, restoration of load-bearing capacity, and prevention of cartilage degeneration in the joint. In addition, implantation strategies and fixation methods (e.g., suturing or press-fit approaches) will need to be optimised to ensure stable positioning of the graft during early healing. The immunological response to implanted constructs, particularly when using allogeneic cell sources or non-native materials, must also be carefully evaluated.

From a manufacturing perspective, translation will require the development of GMP-compliant workflows for MSC expansion, μ T production, and biofabrication, ensuring reproducibility, sterility, and scalability. This includes establishing closed-system bioreactor platforms and standardised protocols for cell sourcing, expansion, and differentiation. Furthermore, regulatory considerations will necessitate a clear definition of the product classification (e.g., combination product involving cells, biomaterials, and biologics), as well as demonstration of safety, efficacy, and batch-to-batch consistency.

Finally, strategies for clinical implementation must consider whether autologous or allogeneic approaches are most feasible. While autologous therapies minimise immune risk, they are time- and cost-intensive, whereas allogeneic approaches require robust strategies to ensure immune compatibility and safety. Addressing these challenges will be essential to enable successful translation of this bioprinting platform from laboratory-scale development to clinical application.

Overall, successful clinical translation will require coordinated advances in preclinical validation, scalable manufacturing, and regulatory-compliant material and cell processing.

8.2 Conclusions

- 1) Early-stage μ Ts support strong chondrogenic differentiation, and increasing μ T density enables the fabrication of cartilage grafts at clinically relevant sizes.
- 2) A convergent bioprinting platform enabled extrusion of MSC-derived μ Ts into a support bath to engineer cartilaginous tissues with user-defined collagen network architectures.
- 3) A versatile 4D bioprinting approach, using temporally adapting support baths, modulated the phenotypic response of MSC μ Ts and supported the development of highly anisotropic tissues such as articular cartilage, meniscus, and ligament.

- 4) Blending XG with XG-MA balanced the need for the support bath to act as a physical boundary during critical stages of μ T and cell maturation, coupling suitable rheology with controlled degradability to enhance inter-filament fusion in scaled grafts.
- 5) Spatial growth factor patterning with dual bioinks provided regional phenotype control, enabling the fabrication of zonally defined, meniscus-like tissues.

Overall, these elements together formed a coherent framework for engineering large, structurally organised, and functionally specialised grafts with clear translational potential.

Supporting Information

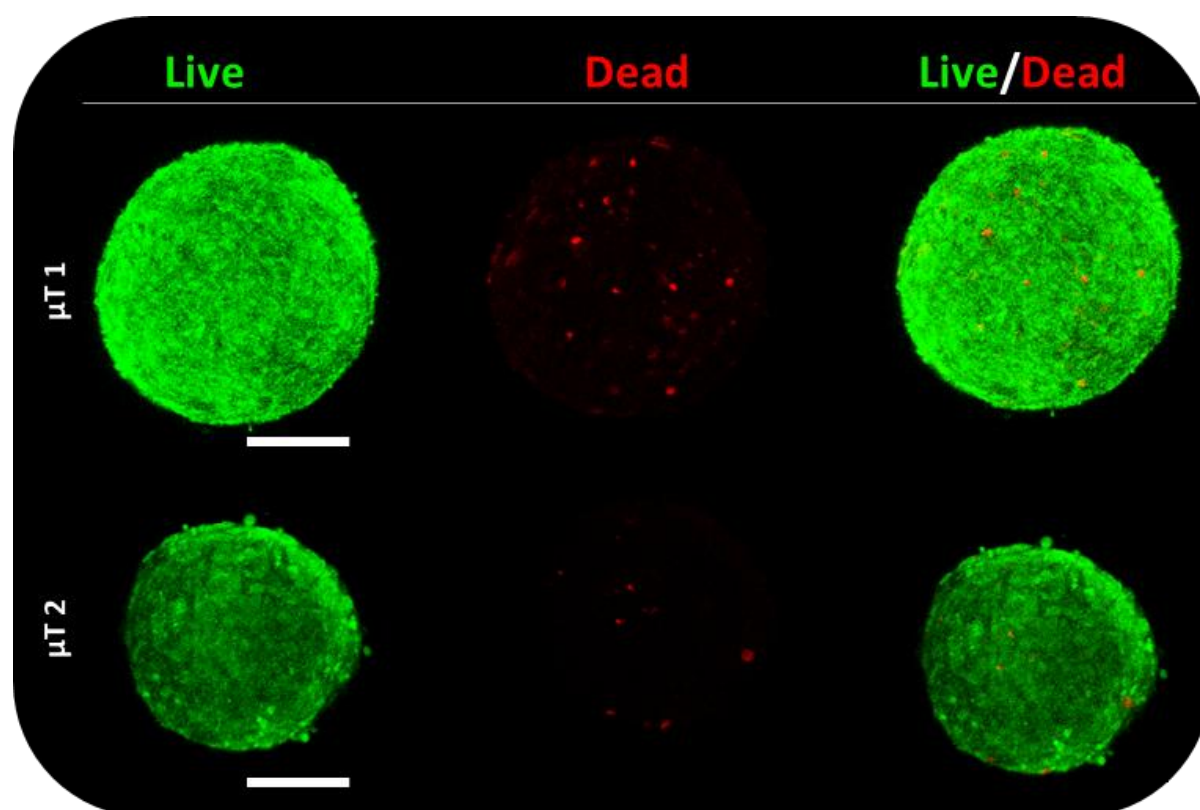


Fig. S3.1. Single μ Ts show high viability when cultured for 2 days in CDM in high high throughput microwell system. Viability assessment of the single μ Ts after 2 days in chondrogenic media cultured in 5 mL of CDM with a final density of 7.5×10^3 cells/mL. Most of the μ Ts were positive for live cells stained in green for Calcein-AM and dead cells stained in EthD-1. Only 2 samples are shown as representative images. Scale bar: 100 μ m.

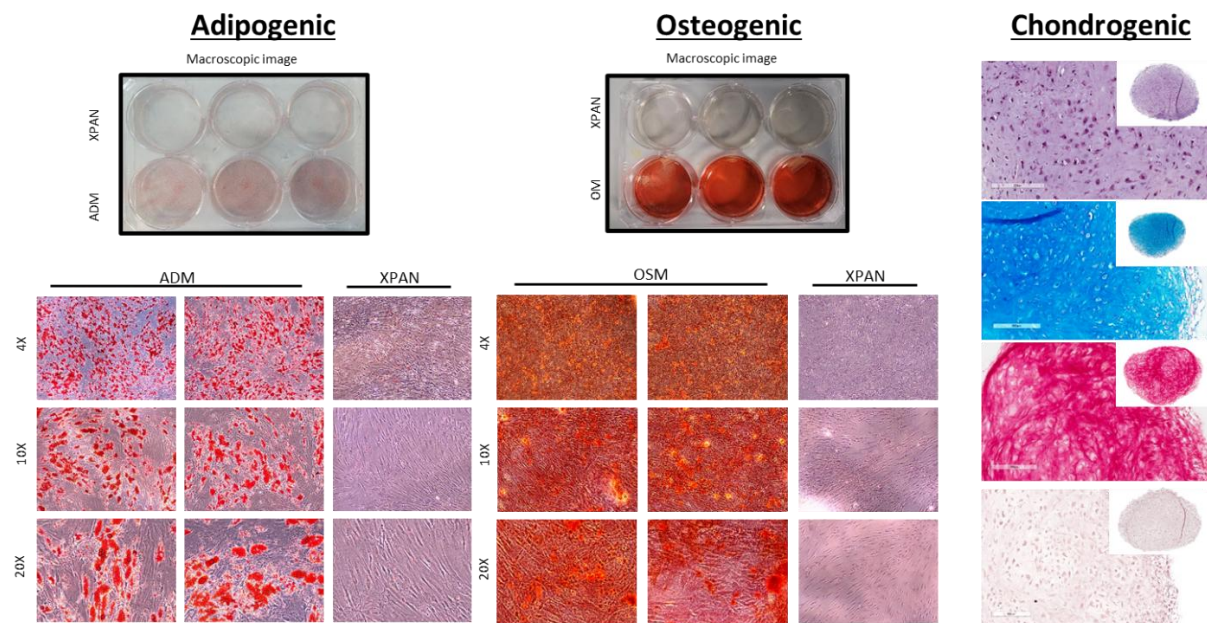


Figure S3.2. Trilineage differentiation of bone marrow–derived MSCs. (A) Adipogenic differentiation of MSC monolayers cultured for 21 days in adipogenic differentiation medium (ADM) or maintained in expansion medium (XPAN, control). Top: macroscopic images of 6-well plates showing triplicate wells per condition. Bottom: representative micrographs of Oil Red O–stained cultures at 4×, 10×, and 20× magnification, highlighting intracellular lipid droplet accumulation in ADM-treated cells. (B) Osteogenic differentiation of MSC monolayers cultured for 21 days in osteogenic medium or XPAN (control). Top: macroscopic images of 6-well plates with triplicate wells per condition. Bottom: representative micrographs of Alizarin Red–stained cultures at 4×, 10×, and 20× magnification, showing calcium-rich mineralised matrix in osteogenic cultures. (C) Chondrogenic differentiation in 3D pellet culture after 21 days in chondrogenic medium. Pellets were sectioned and stained with H&E (general morphology), Alcian Blue (sGAG-rich cartilage matrix), Picrosirius Red (collagen), and Alizarin Red (mineralisation). Scale bar = 100 μ m.

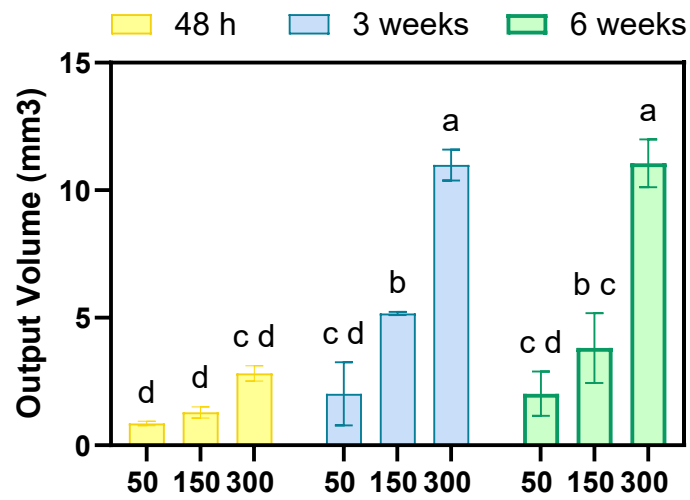


Figure S3.3. Estimated output volume of fused μ T constructs. Construct volumes were calculated from measured diameters in brightfield images, assuming spherical geometry. Based on average construct diameters of ~ 1.8 mm and ~ 2.8 mm, the corresponding theoretical volumes were ~ 3.05 mm³ and ~ 11.49 mm³, respectively. Data are shown for constructs formed from 50, 150, and 300 μ Ts across different time points, with variability reflecting differences in construct size and morphology. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. Results are presented using a compact letter display due to multiple comparisons, where groups sharing the same letter are not significantly different.

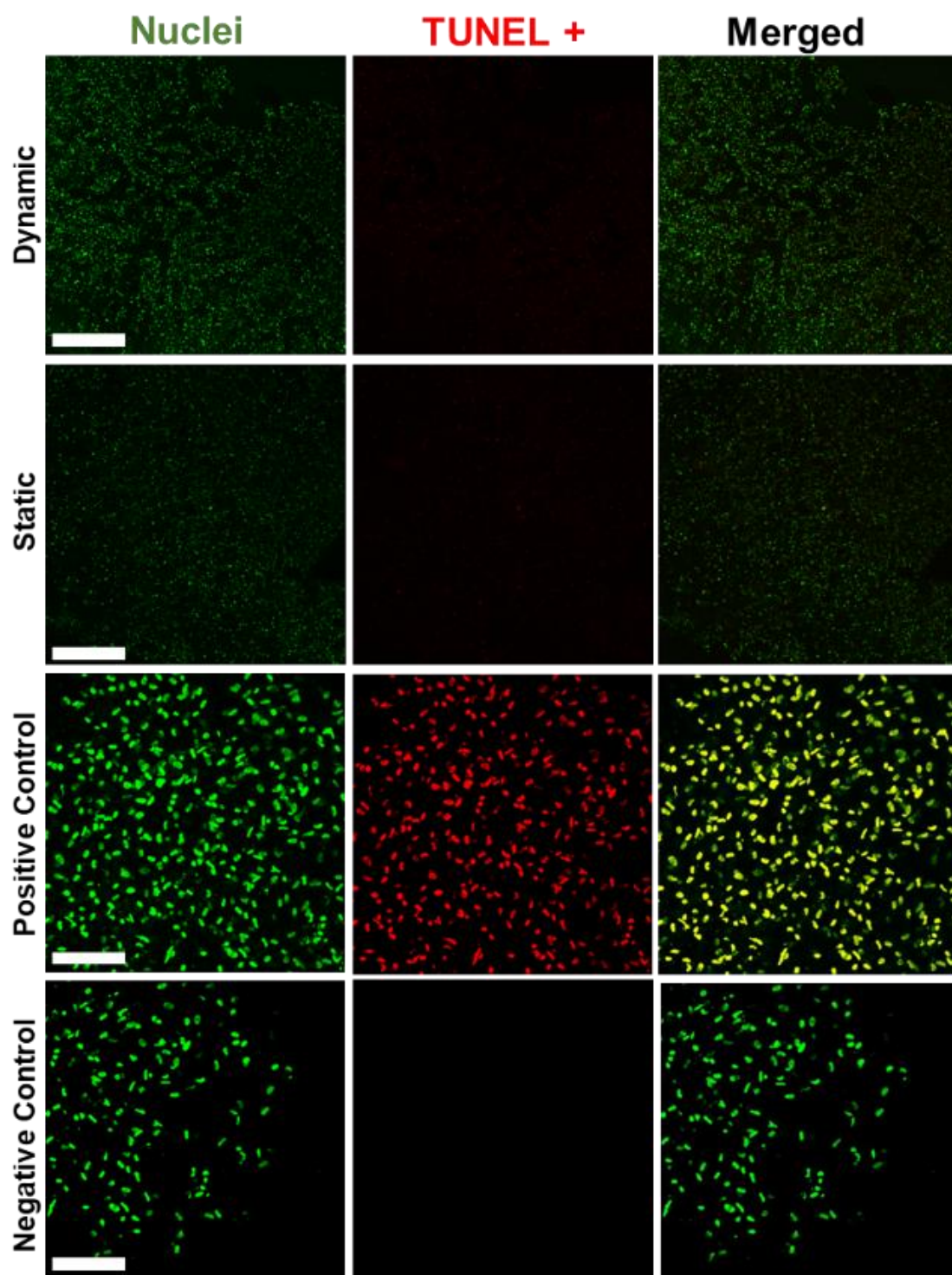


Figure S3.4. TUNEL staining for apoptosis. Representative images of the core of a scaled-up graft from caprine MSCs stained with a green nuclear stain and a red TUNEL stain. TUNEL-positive apoptotic cells are those in which the green and the red colocalise to the same cell. Scale bar: 200 μ m.

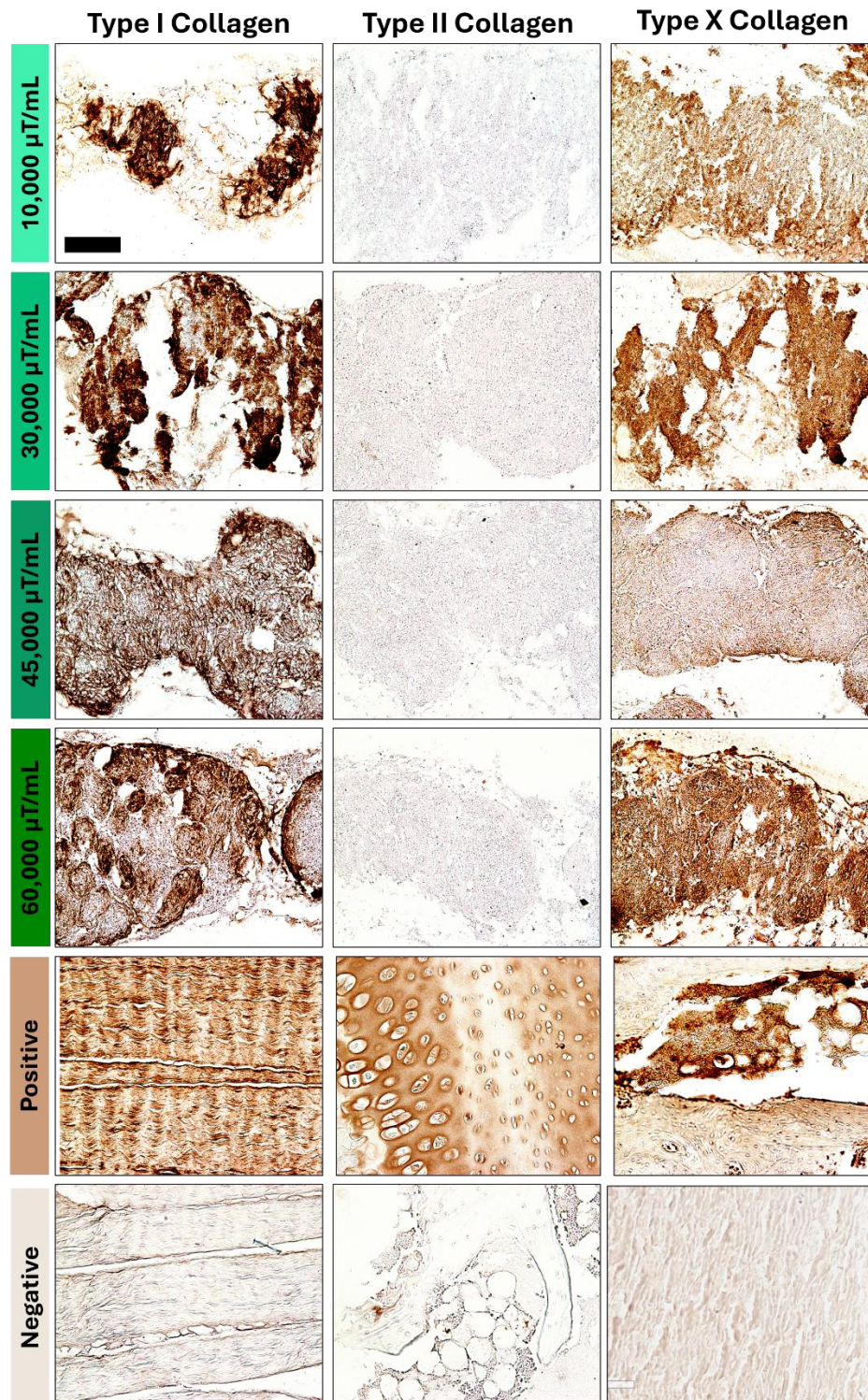


Figure S 4.1. Xanthan gum support bath does not favour chondrogenesis but fibrochondrogenesis and hypertrophy. DAB immunochemistry for collagen type I, II and X. Isotype controls included ligament sections with and without primary antibody for collagen type I; AC as a positive control for collagen type II and bone as a negative control for collagen type II; bone as a positive control for collagen type X; and AC as a negative control for collagen type X. Scale bar: 200 μm .

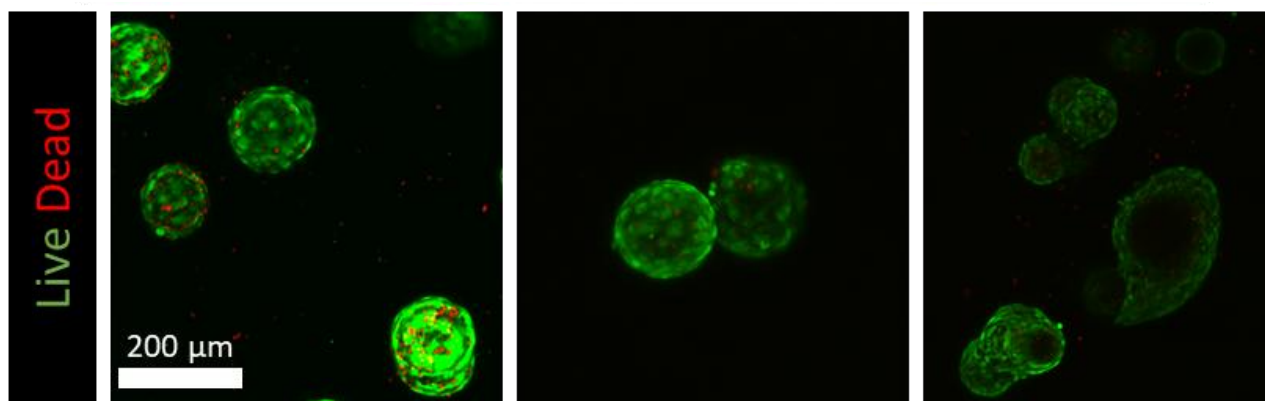


Figure S4.2. Microtissue viability post-encapsulation with gelatin prior to bioprinting. Live/dead of μ Ts (2000 cells/ μ T) encapsulated in gelatin 1% prior to printing show high viability of μ Ts. Green: Calcein (Live cells), Red: Ethidium Bromide (dead cells). Sb: 200 μ m.

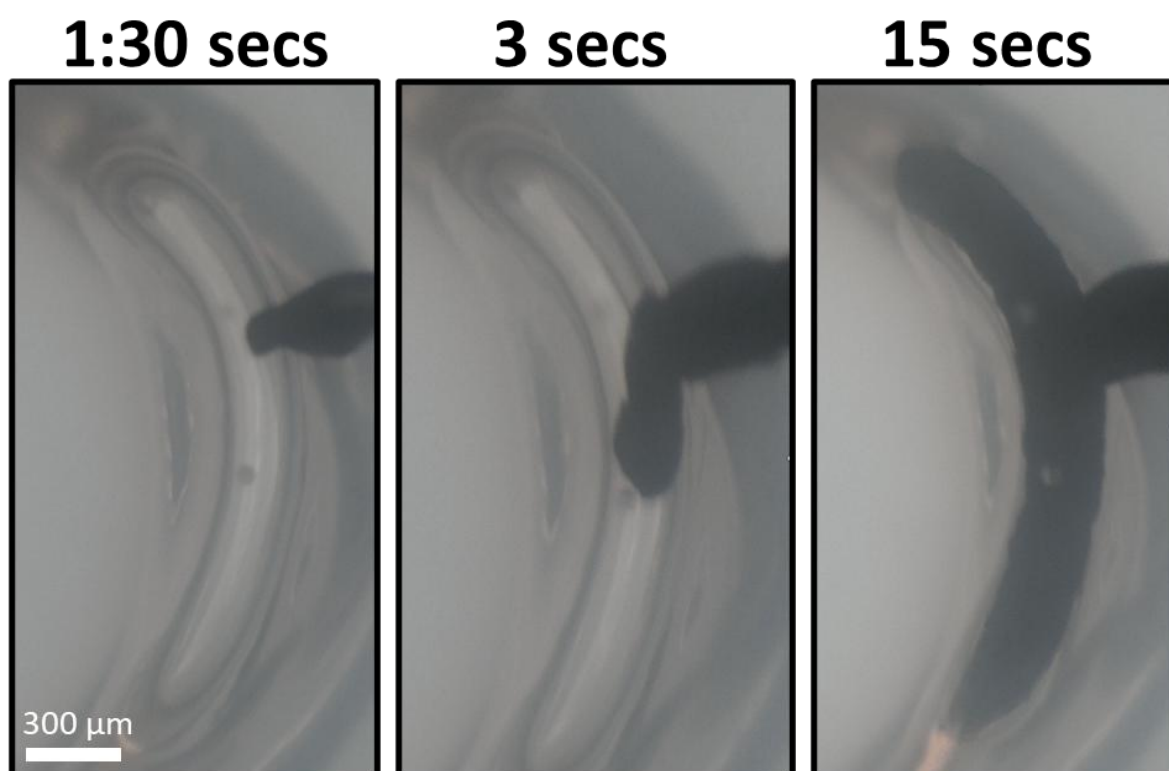


Figure S 5.1. Perfusion of black dye through a void channel formed after the diffusion of a printed 1% gelatin filament within the microenvironment. Following printing and UV curing of the support bath, the gelatin bioink liquefied and diffused into the surrounding microenvironment, leaving behind a void channel. This channel was subsequently perfused with black dye to confirm that the channel remained open, illustrating how gelatin diffusion supports the temporal reduction in filament diameter over time. Scale bar: 300 μ m.

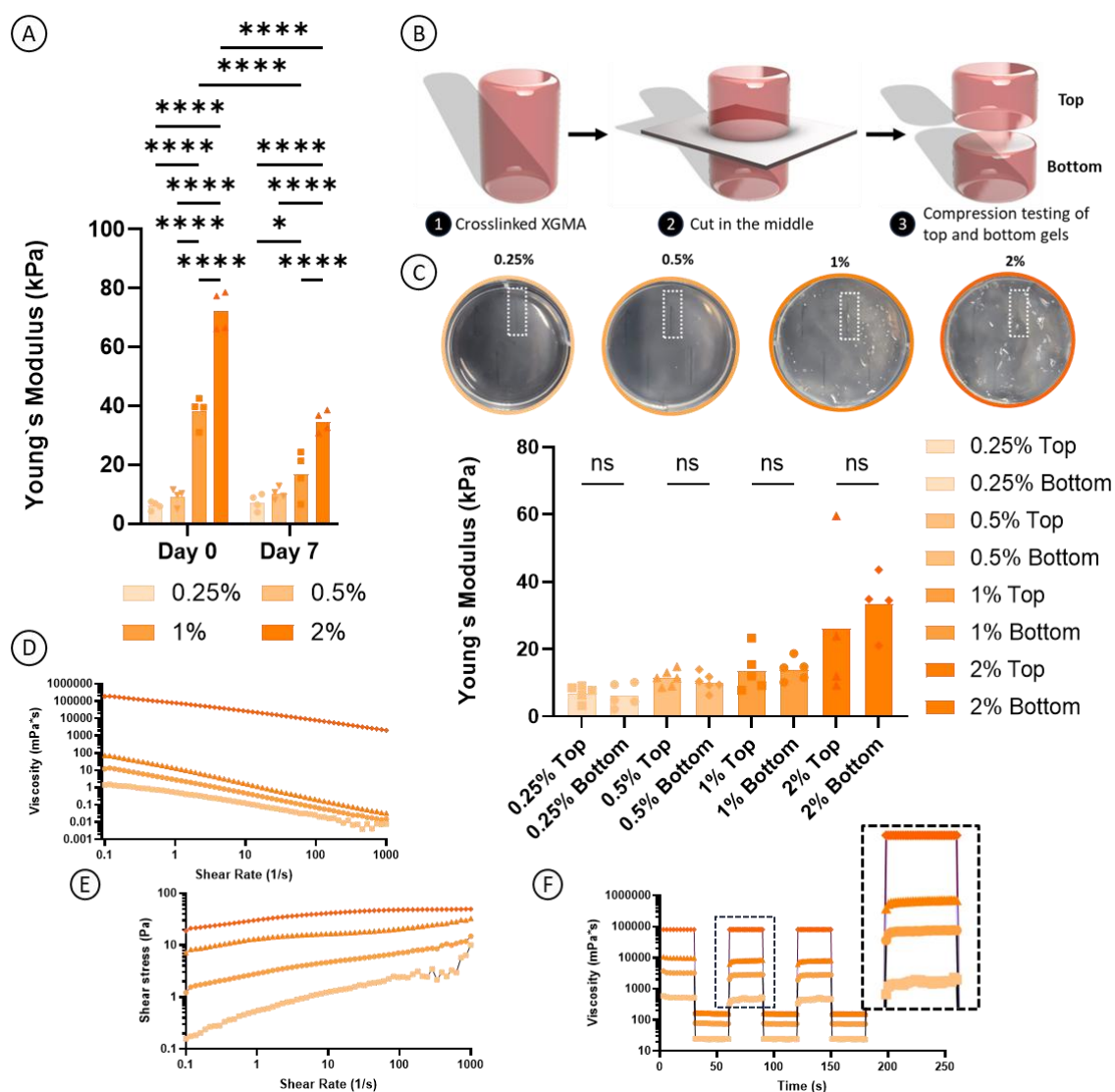


Figure S 5.2. Rheological properties of XG-MA at different concentrations and UV-curing depth on the bath stiffness. A) Compression mechanical testing of XG-MA gels at day 0 and day 7 under physiological conditions. A two-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. B) Graphical representation of mechanical testing of both halves (top and bottom) of the support bath post UV exposure to assess UV curing depth C) Macroscopic pictures of acellular (1% gelatin only) printed filaments into support baths at different concentrations, with different optical properties. Mechanical testing of both halves (top and bottom) of each gel to assess curing depth dependency of all concentrations of the support bath post-UV exposure (4 min). A two-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. ns: non-significant. D) Viscosity dependency on shear rate of the support bath at different concentrations, from low shear rate (0.1 s^{-1}) to very high shear stress (1000 s^{-1}) demonstrating shear-thinning properties E) Shear stress dependency on shear rate of the support bath at different concentrations, from low (0.1 s^{-1}) to high (1000 s^{-1}) shear stress F) Stress-

relaxation behaviour of the XG-MA support bath at different concentrations to assess the recovery of the material post applying high shear stress

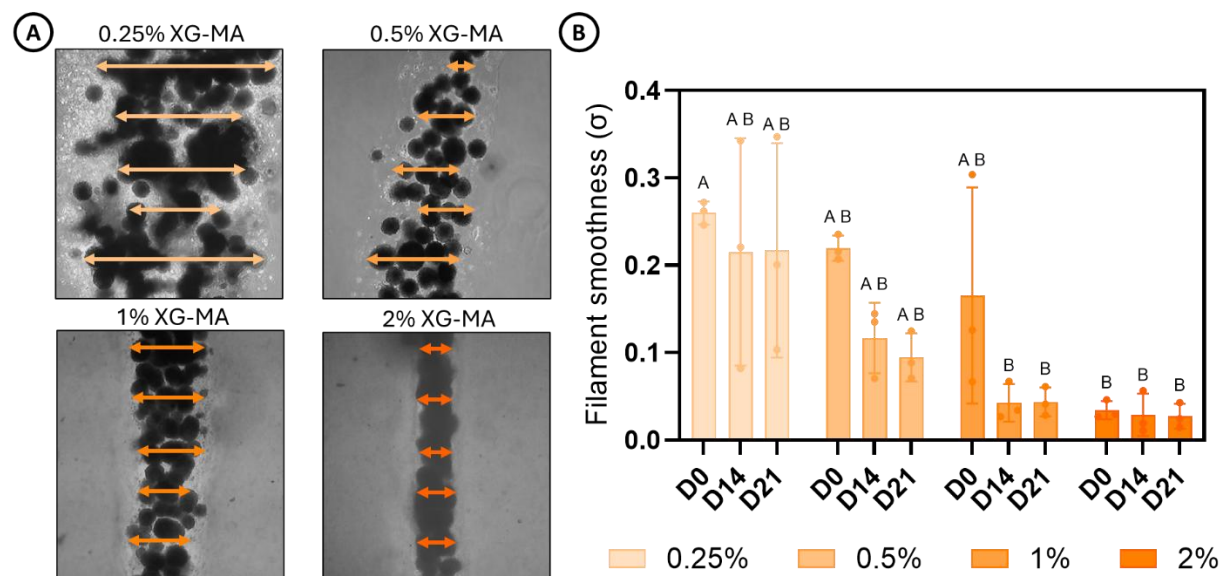


Figure S 5.3. Quantification of filament smoothness. A) Brightfield microscopy images were used to assess local diameter variations of printed μ T filaments. For each filament, five diameter measurements were taken at distinct regions along its length ($n = 3$ images per condition). The brightfield images represent the printed μ Ts at day 0 under different conditions (refer to Fig. 1). B) The standard deviation of these measurements was calculated as an index of smoothness, with lower values indicating more uniform and smoother filaments, and higher values reflecting increased irregularity. This parameter helps assess μ T fusion and filament compaction post-printing. 2-way ANOVA followed by Tukey's post hoc comparison test was performed to assess the differences between the groups; significance was accepted when $p < 0.05$. Statistically comparable groups are labelled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

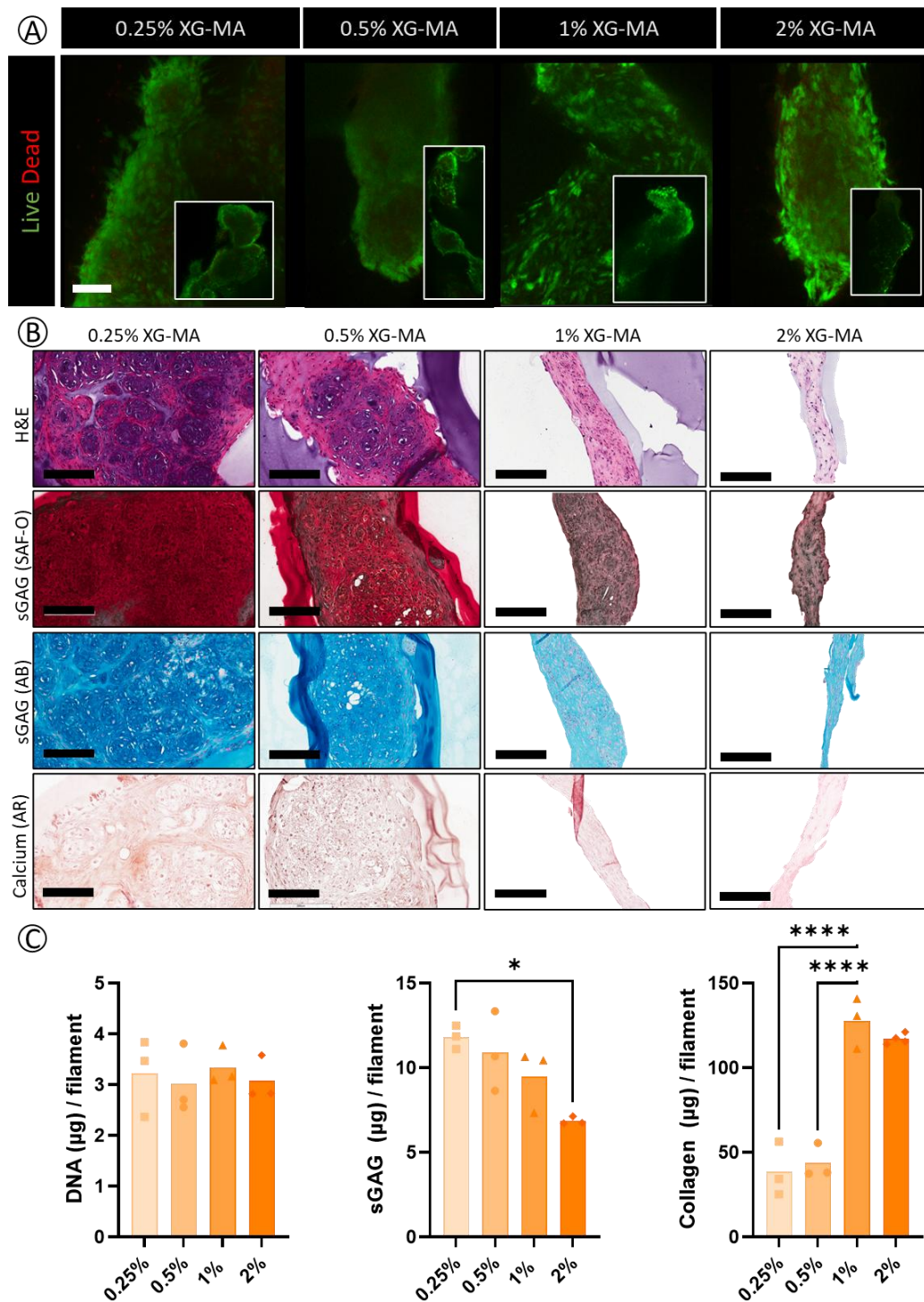


Figure S5.4. Stiffness of the supporting bath post printing influences the deposition of sGAGs and matrix re(modelling) over time in culture. A) Live/dead staining of printed μ Ts into XG-MA support bath, 7 days after printing. Green: calcein AM; Red: Ethidium Bromide. Sb: 100 μ m. B) Histological evaluation of the printed μ Ts after 28 days in culture with H&E, Safranin-O (SAF-O) and Alcian Blue to evaluate sGAGs deposition, which decreases with the increase in stiffness of the XG-MA support bath. Sb: 200 μ m. C) Biochemical quantification of total DNA, sGAG and collagen per printed filament. Statistical difference is determined using a two-way

ANOVA test with Tukey's post hoc comparison test. Significance was accepted with $p < 0.05$. * Indicates $p < 0.05$, *** indicates $p < 0.0001$.

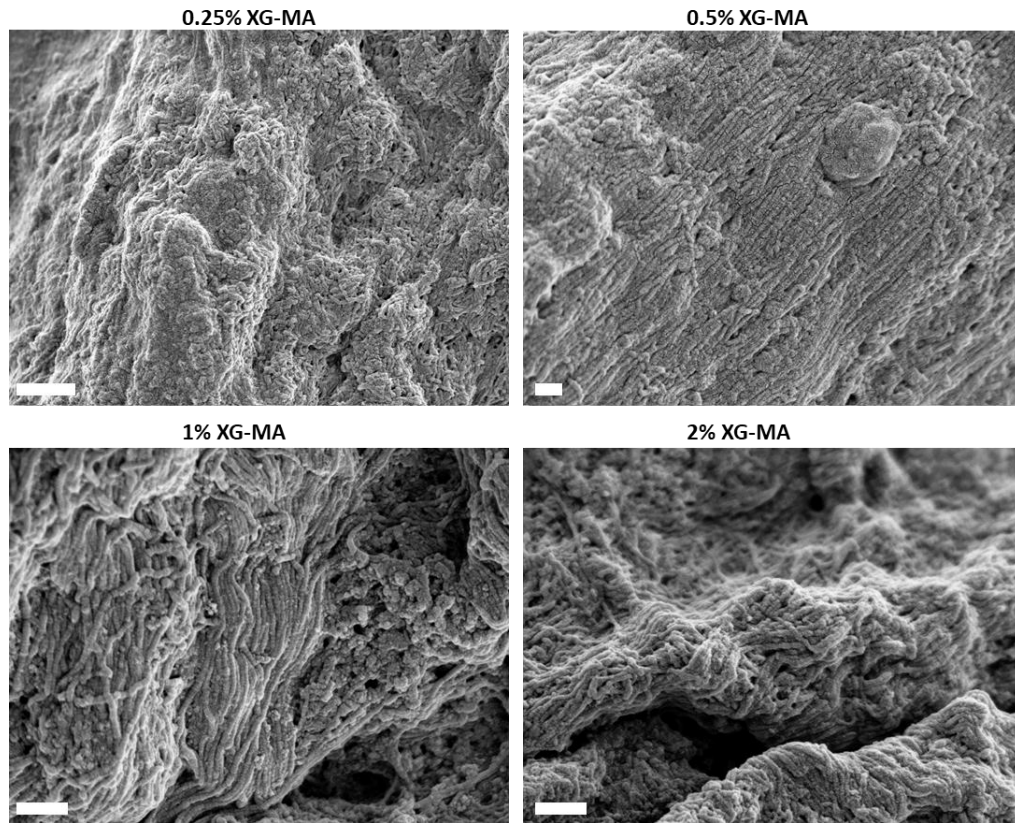


Figure S5.5. (Re)modelling of the collagen fibers along the printing direction after 28 days in culture. Scanning electron microscope (SEM) pictures of bioprinted μ Ts to show the ECM organization and (re)modelling, driving tissue maturation and differentiation. Sb: 10 μ m.

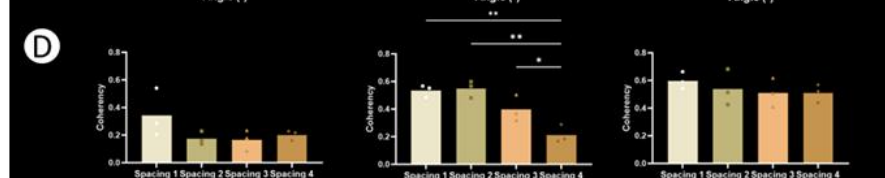
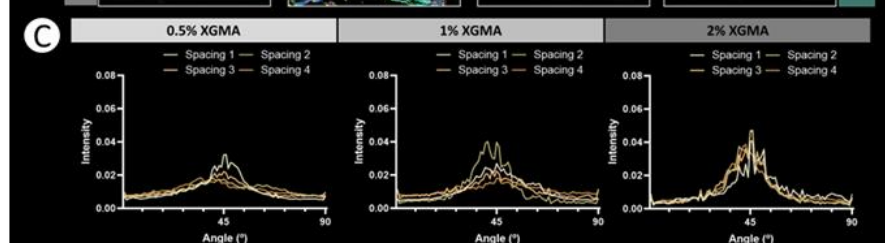
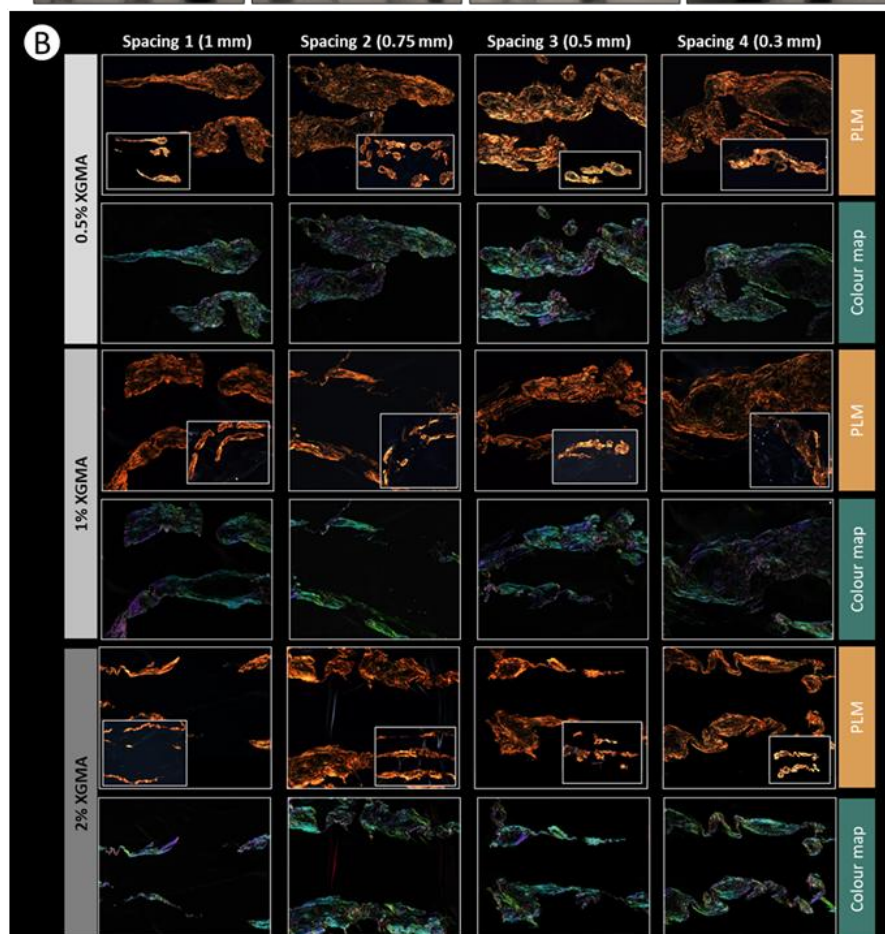
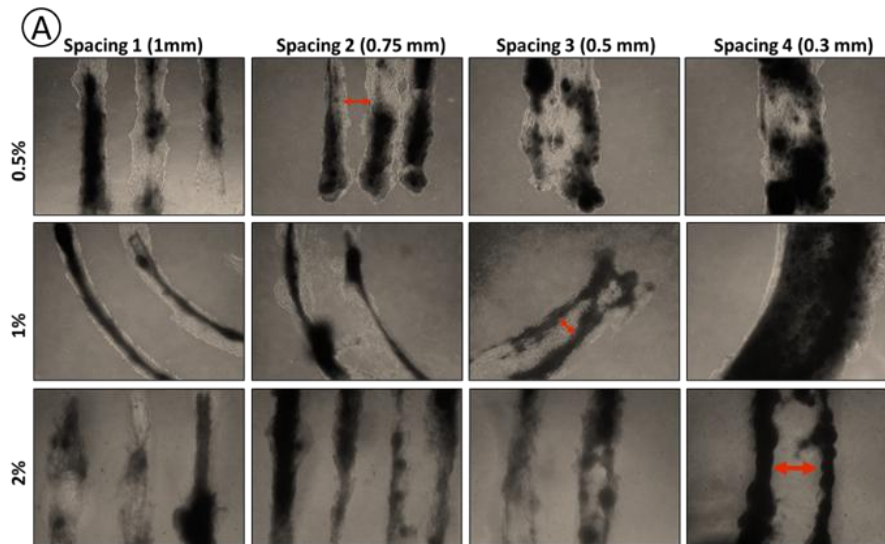


Figure S5.6. The spacing (distance) between filaments post-printing modulates the anisotropy of a scaled-up bioprinted tissue construct. A) Brightfield microscope pictures of bioprinted μ Ts 28 days post printing, showing fusion of the μ Ts and adequate distance between each filament. B) Polarised Light Microscope images of bioprinted filaments at different distances after 28 days of culture in TGF- β 3-supplemented media, in a support bath with variable stiffness. C) The mean average of the collagen fibres indicates that the spacing of the filaments into a scaled-up construct influences the final anisotropy of the printed construct. D) Fibre coherency shows highly aligned tissues. Colour maps are generated from PLM images. Here, colour hue is used to indicate fibre orientation, where blue/cyan indicates fibres oriented at 0 degrees and pink/red indicates fibres oriented at 90 degrees. For all the graphs, a one-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. * Indicates $p < 0.05$. **Indicates $p < 0.01$. For all the graphs: $n = 3$.

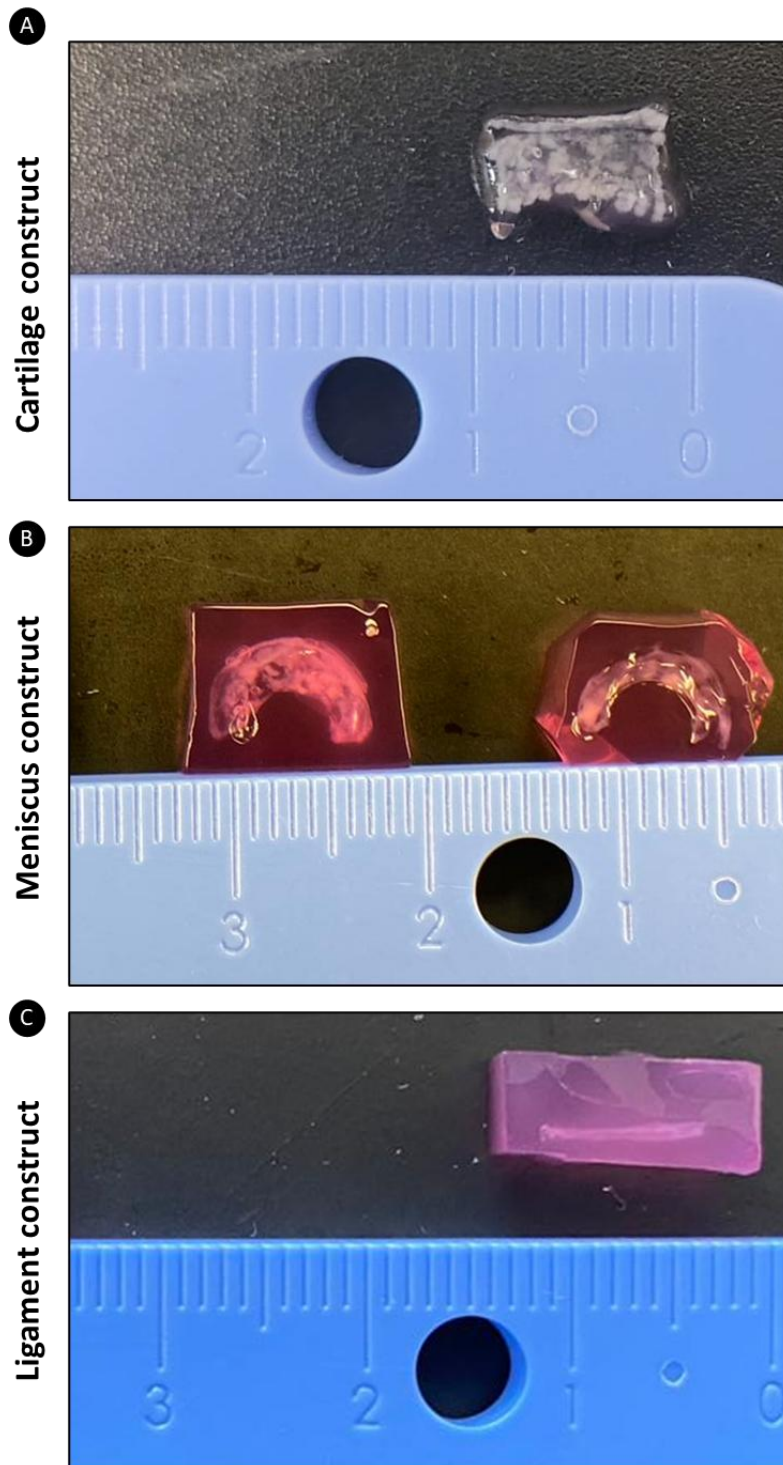


Figure S5.7. Macroscopic images of scaled-up tissue grafts. A) Cartilage construct B) Meniscus constructs C) Ligament tissue construct.

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Nomenclature

The nomenclature contains some of the abbreviations used in this thesis. It is not a full list of all abbreviations and symbols used – they are explained in the text whenever used.

μT	Microtissue
4D	Four-Dimensional
AC	Articular Cartilage
ACAN	Aggrecan
ACI	Autologous Chondrocyte Implantation
AM	Additive Manufacturing
ANOVA	Analysis of Variance
aOBbs	anisotropic Organ Building Blocks
BMP	Bone Morphogenetic Protein
cABC	Chondroitinase ABC
CDM	Chondrogenic media
CHAD	Chondroadherin
COL1	Collagen type I
COL10	Collagen type X
COL2	Collagen type II
COMP	Cartilage oligomeric matrix protein
CT	Computer Tomography
CTGF	Connective Tissue Growth Factor
DE	Developmental Engineering
DMEM	Dulbecco`s Modified Eagle`s Medium
DMMB	Dymethylmethylene Blue
DMSO	Dimethyl Sulphoxide
DPL	Digital Light Processing
ECM	Extracellular Matrix
EO	Ethylene Oxide
FBS	Fetal Bovine Serum
FDM	Fused Deposition Modelling

FGF	Fibroblasts Growth Factor
FLight	Filamented Light Biofabrication
FRESH	Freeform Reversible Embedding of Suspended Hydrogels
GelMA	Gelatin Methacryloyl
H&E	Haematoxylin & Eosin
Hg	High glucose
iPSCs	induced Pluripotent Stem Cells
MEW	Melto Electro Writing
MMP	Matrix Metallo Proteinase
MPCs	Meniscus Progenitor Cells
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MSCs	Mesenchymal stem/stromal cells
OA	Osteoarthritis
OMI	Optical Modulation Instability
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
PLGA	Poly (lactic-co-glycolic acid)
RGD	Arginine-Glycine-Aspartic Acid amino acid sequence
ROI	Region of Interest
sGAG	Sulphated Glycosaminoglycan
SLAM	Suspended Layer Additive Manufacturing
Sox9	Sry-box Transcription Factor 9
SWIFT	Sacrificial Writing into Functional Tissue
TAZ	Transcriptional co-activator with PDZ- Binding motif
TE	Tissue Engineering
TGF- β	Transforming Growth Factor Beta
TJA	Total Joint Arthroplasty

VEGF

Vascular Endothelial Growth Factor

YAP

Yes Associated Protein

List of Figures

Figure 2.1. Specific collagen organisation in different musculoskeletal tissues. (A) Polarised light microscopy (PLM) images of articular cartilage stained with picrosirius red, showing the superficial (STZ, pink), middle (MZ, green) and deep (DZ, blue) zones, each accompanied by corresponding helium ion microscopy images. (B) PLM image of collagen fibres in native meniscal tissue with corresponding higher-magnification transmission electron microscopy (TEM) images of collagen fibres. (C) PLM image of the anterior cruciate ligament (ACL) with corresponding scanning electron microscopy (SEM) images showing collagen bundles. The image was adapted from [89-93].

Figure 2.2 Microtissues are widely used as biological building blocks due to their capacity to fuse and self-organise. (A) Microtissues are introduced into a cartilage explant, where they fuse and develop into a biomimetic tissue that is well integrated with the native explant. (B) Microtissue self-organisation is guided by the design of polymeric microchambers, enabling the engineering of aligned cartilaginous microtissue-based constructs. The image was adapted from [64, 164].

Figure 2.3. Meniscus-derived cells are isolated and used to generate fibrocartilaginous microtissues for meniscus tissue engineering. (A) Mesenchymal stromal cells (MSCs) obtained from meniscal tissue debris are isolated and self-assembled into pellets that undergo chondrogenic differentiation, as shown by sGAG staining and collagen type I staining. (B) Meniscus progenitor cells isolated from meniscal debris are used to recreate zonally organised meniscal tissues, exhibiting phenotypical differences between the outer and inner zones. The image was adapted from [78, 166].

Figure 2.4. Bioprinting strategies for the engineering of anisotropic tissues via physical cue guidance. A) Schematic illustrating how imposed boundary conditions can be used to direct the self-organisation of cells or cell aggregates during tissue maturation. B) MSCs are deposited by inkjet bioprinting into polymeric microchambers that act as boundary conditions; the printed cells assemble into aggregates, adhere to the polymer surface and remodel the newly secreted ECM along these physical cues. C) In a FLight bioprinting approach, cells are encapsulated within a photoresin (i.e. GelMA) and, via OMI, boundary conditions are created at the microscale that guide cell alignment. D) Bioprinting of skeletal muscle constructs using physical constraints to generate functional grafts with enhanced anisotropy. The image was adapted from [45, 81, 206, 207].

Figure 2.5. Schematic representation of key parameters for microtissue bioprinting. (1) Control of microtissue size reduces shear stress during extrusion, thereby preserving cell viability and internal organisation. (2) The density of microtissues within the cartridge before printing determines the extent of post-printing

microtissue contact and fusion. (3) Bioink degradability governs microtissue fusion during maturation: a fast-degrading or sacrificial ink promotes microtissue–microtissue contact, fusion, ECM remodelling and formation of a cohesive construct, whereas a slowly degrading bioink limits contact between microtissues, leading to maturation of individual microtissues but a non-cohesive overall construct. Figure created with Autodesk Fusion.

Table 1. Comparison of common embedded bioprinting support bath systems and their suitability for μ T bioprinting

Figure 3.1. MSC-derived μ Ts secrete a cartilaginous matrix over 28 days of culture in chondrogenic media.

(A) Experimental settings: μ Ts are cultured in agarose micro-moulds. (B) Histological panel for matrix components after 2, 4, 7 and 28 days in chondrogenic media. (C) Biochemical quantification of the μ Ts; levels of DNA, sGAG, and collagen are normalised to the number of μ Ts and to the DNA content. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, when tested using a one-way ANOVA ($n=3$) followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups. Scale bar is 60 μ m. Fusion 360™ was used to design the graphical abstract, and BioRender was used for the layout of the final figure.

Figure 3.2. μ Ts of differing maturation levels show differences in fusion kinetics, final size and collagen deposition over 28 days of culture in chondrogenic media.

A) Microtissues derived from human bone marrow were cultured separately in agarose microwells for 2, 4, and 7 days (FD-2, FD-4, and FD-7, respectively). Following this individual culturing period, the microtissues were fused to create a larger tissue. All samples were assessed through histology, immunohistochemistry, biochemistry, and qPCR. B) Brightfield microscope images depict fused μ Ts observed over a 21-day period in culture in chondrogenic media, followed by C) diameter measurements. Scale bar: 500 μ m. D) Samples were subjected to picrosirius red staining to visualise the collagen present within the tissue. The arrows point to remnants of the structure of the individual μ Ts. Scale bar: 200 μ m. Significant differences are denoted as: ** $p < 0.01$, when tested using a two-way ANOVA test ($n=20$) followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups. F-D2: μ Ts fused at day 2 maturation level. F-D4: μ Ts fused at day 4 maturation level. F-D7: μ Ts fused at day 7 maturation level.

Figure 3.3. Cell-cell and cell-ECM interactions during μ T fusion are dependent on their maturation levels.

N-Cadherin and Vimentin are stained with respective monoclonal antibodies, and nuclei are stained with Hoechst staining. Immunofluorescence quantification was performed by normalising the region of interest (ROI) with the number of nuclei in the same ROI. mRNA expression of the cell-cell and cell-ECM markers, vimentin (VIM) and N-Cadherin (CDH2), were analysed by real-time PCR. Samples were normalised with MSCs

in monolayer. A) N-Cadherin staining, immunofluorescence quantification (left graph) and mRNA expression (right graph) for fused μ Ts at different maturation levels over 7 days. B) Vimentin staining, immunofluorescence quantification (left graph) and mRNA expression (right graph) for fused μ Ts at different maturation levels over 7 days. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a two-way ANOVA test ($n=6$) followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups. F-D2: μ Ts fused at day 2 maturation level. F-D4: μ Ts fused at day 4 maturation level. F-D7: μ Ts fused at day 7 maturation level. Scale bar: 200 μ m.

Figure 3.4. Chondrogenic gene expression following μ T fusion. mRNA expression of chondrogenic markers from left to right: COL1A1, COL2A1, ACAN and SOX9 analysed by real-time PCR and normalised against the housekeeping gene 18s rRNA. Each sample was normalised against the negative control of monolayer cells cultured for 2 days. F-D2: μ Ts fused at day 2. F-D4: μ Ts fused at day 4. F-D7: μ Ts fused at day 7. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a two-way ANOVA test ($n=6$) followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups.

Figure 3.5. Fusion of μ Ts at day 2 supports cartilage tissue formation. A) Histological panel for standard chondrogenic markers: haematoxylin and eosin (H&E) staining, alcian blue staining, alizarin red staining, immunohistochemistry for collagen type I and collagen type II) after 28 days in chondrogenic media (scale bar: 250 μ m). Last column is a brightfield microscope image of fused μ Ts at 28 days in chondrogenic media. B) Biochemical quantification of the μ Ts, levels of sGAG and collagen normalised to DNA content. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a one-way ANOVA test ($n=3$) for biochemical analysis and a two-way ANOVA for diameter measurements, followed by Tukey's multiple comparison test to assess the difference in the gene expression between groups. F-D2: μ Ts fused at day 2 maturation level. F-D4: μ Ts fused at day 4 maturation level. F-D7: μ Ts fused at day 7 maturation level.

Figure 3.6. Increasing the density of μ Ts per fused construct improves chondrogenesis. A) brightfield imaging of fused μ Ts over a period of 42 days in culture in chondrogenic media followed by diameter measurement. Statistical differences were analysed using two-way ANOVA followed by Tukey's post hoc test; however, no significant differences were observed between groups at the same time point. Scale bar: 500 μ m. B) histological panel of fused μ Ts after 42 days in culture for standard chondrogenic markers: haematoxylin and eosin (H&E) staining, Alcian blue staining (AB), alizarin red staining (AR) and picosirius red staining (PSR). Scale bars: 200 and 400 μ m. C) Biochemical quantification for chondrogenic markers (sGAG, Collagen). D) Immunohistochemistry for collagen types I, II and X. Significant differences are denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when tested using a one-way ANOVA test ($n=3$) followed by Tukey's

multiple comparison test to assess the difference in the gene expression between groups. μ T: μ Ts. Scale bars: 300 and 400 μ m.

Figure 3.7. Engineering of scaled-up grafts using cartilage μ Ts. A) Stereomicroscopy (macro) images of a scaled-up engineered construct after 42 days of chondrogenic induction (scale bar: 2 mm); set up for tensile testing of fused μ Ts after 42 days in culture using a tensile biaxial tester (CellScale). B) Tensile modulus quantification of fused μ Ts after 42 days in culture in CDM. Biochemical quantification of constructs after 42 days of culture. Statistical analysis using a t-test revealed no significant differences between groups ($p > 0.05$). C) Histological and immunohistochemical panel of chondrogenesis markers: H&E, Alcian Blue, Alizarin Red, Picrosirius Red, collagen 1 (COL I), collagen 2 (COL II), collagen 10 (COL X). Scale bar: 400 μ m and 2 mm.

Figure 4.1. Bioinks characterisation. A) Viscosity and shear stress profiles of 2% methylcellulose and 5% gelatin measured over a shear rate range of 0–1000 s^{-1} at 4 °C. B) Representative prints of bioinks in a grid pattern (scale bar: 100 μ m). μ T-free: bioinks printed without cellular components. μ T-laden: bioinks containing μ Ts at a density of 10,000 μ Ts/mL. μ T viability was evaluated 7 days post-printing. C) Filament diameter and spreading ratio (D) of printed filaments containing μ Ts. E) Percentage of live cells relative to total cells, quantified using ImageJ. Live cell percentage was normalised to the total cell percentage.

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Figure 4.3. Printability of gelatin at different concentrations. A) Brightfield microscope pictures of gelatin bioink bioprinted in a grid pattern on a flat surface. Scale bar: 200 μ m. B) Viscosity and C) shear stress profiles of gelatin bioinks measured over a shear rate range of 0–1000 s^{-1} at 4 °C. D) Stress ramp curves for gelatin bioinks at different concentrations. E) Yield stress of gelatin bioinks. F) Brightfield microscope pictures of 1% gelatin printed into the xanthan gum support bath at different needle gauges. G) brightfield microscope pictures of 1% gelatin mixed with a particle-based ink at various densities (low, medium, high). H) Filament diameter measurements of 1% gelatin printed into XG support bath with different needle gauge (referred to panel F), and I) filament diameter of 1% gelatin mixed with particle-based ink at various densities (referred to panel G).

Figure 4.4. μ Ts were extruded into a support bath at varying densities, showing improved fusion at higher densities. A) Macroscopic images of μ T-laden bioinks at increasing densities within the printing cartridge, with corresponding brightfield images of printed μ Ts arranged in a filament pattern at day 0 and day 3, highlighting fusion over time. B) Filament diameter measurements at day 0 and DNA quantification by biochemical assays compared to nozzle diameter (grey). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$). C) Histological staining for haematoxylin & eosin (H&E), sGAG (AB), collagen (PR), and calcium deposits (AR). Scale bar: 400 μ m.

Figure 4.5. Xanthan gum (XG) and methacrylate xanthan gum (XG-MA) were successfully synthesised, and they show good rheological properties suitable for support baths. A Schematic of XG modification with glycidyl methacrylate (created with BioRender). B) ^1H -NMR spectra of xanthan and methacrylate XG. The peaks highlighted with the red arrows correspond to the peaks (6.58 and 6.17 ppm) of the methacrylate group attached to the XG backbone. C) Viscosity and D) shear stress profiles XG-based baths measured over a shear rate range of 0–1000 s^{-1} at room temperature. E) Viscosity profiles measured at temperatures between 4–38 $^{\circ}\text{C}$, showing stable viscosity across conditions, confirming suitability for printing at physiological temperatures to support cell viability. F) Viscosity profiles under low (0 s^{-1}) and high (1000 s^{-1}) shear rates across multiple cycles, demonstrating the thixotropic behaviour of the baths.

Figure 4.6. XG-MA support bath limits the contraction of μ T-laden bioprinted constructs and exhibits enhanced chondrogenesis compared to XG. A) Brightfield images of μ Ts printed in XG and XG-MA support baths immediately after printing and after 21 days of culture (45,000 $\mu\text{T}/\text{mL}$) (Scale bar: 200 μm), alongside histological staining for H&E, sulphated glycosaminoglycans (sGAG), collagen, and calcium deposition. Scale bars: 500 and 100 μm . B) Macroscopic images of printed μ T filaments after 21 days in chondrogenic medium. C) Live/dead staining of printed μ Ts stained with Ethidium bromide (Dead) and calcein (Live). Scale bars (from left to right): 300 μm and 50 μm . D) Biochemical quantification of DNA, sGAG content and collagen content related to DNA. Statistical analysis was performed using an unpaired t -test, with significance accepted at $p < 0.05$. (* $p < 0.05$, ** $p < 0.01$).

Figure 4.7. The collagen fibre organisation of printed μ Ts can be better guided using a XG-MA support bath. A) PLM and colour map for the respective PLM image show a comparison of the spatial organisation of the collagen network formed by the μ Ts within the support bath over 4 weeks in culture in chondrogenic media. Sb: 200 μm . B) quantification of the directionality of the collagen fibres within the printed μ Ts and c) fibre coherency, where a value of 1 indicates that fibres are aligned in the same directions, and 0 indicates dispersion of the fibres in all directions. Significant differences were tested using an unpaired t -test. Significance was accepted when $p < 0.05$. D) DAB immunohistochemistry showing collagen types I, II, and X within the printed filaments. Isotype controls included ligament sections with and without primary antibody

for collagen type I; articular cartilage (AC) with and without primary antibody for collagen type II; bone as a positive control for collagen type X; and articular cartilage as a negative control for collagen type X. Sb: 100 μm .

Figure 5.1. The concentration of the support bath influences the extent of filament confinement over time post-printing. A) Graphical abstract of the 4D-bioprinting platform. μT s are fabricated using a high throughput agarose microwell system and cultured for 2 days prior to their harvest and encapsulation into a gelatin bioink and extrusion into a XG-MA support bath. After UV-crosslinking, the filament diameter changes over time due to the changing physical confinement provided by the support bath. B) Young's Modulus of XG-MA hydrogels following UV crosslinking for 4 minutes. C) Viscosity of the un-crosslinked XG-MA bath at different concentrations. D) Yield stress of the un-crosslinked XG-MA bath. E) Brightfield microscope imaging of the printed μT s within XG-MA support baths of differing concentrations at different time points. Filament diameter changes over time for the (F) μT -laden inks and the (G) μT -free inks. Scale bar (Sb): 200 μm . 2-way ANOVA followed by the Tukey's post comparison test was performed to assess the differences between the groups at different time points; significance was accepted when $p < 0.05$. Statistically comparable groups are labeled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

Figure 5.2. The physical properties of the support bath influence MSCs' phenotype. A) YAP1 staining of printed μT s reveals spatial differences in YAP expression depending on the stiffness of the support bath. White arrows indicate regions where YAP colocalises with the nuclei, indicating YAP activation. Positive control is the mouse heart, and negative control is the mouse heart without the primary antibody. B) Expression of a range of genes associated with cartilage (COL2A1, CHAD, COMP, ACAN), fibrocartilage or ligament tissue (COL1A1, TNC, ACTA2). For statistical analysis, a two-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. Statistically comparable groups are labelled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

Figure 5.3. Immunofluorescence staining for specific proteins within the bioprinted μT s. (A) Staining for type I collagen, type II collagen and $\alpha\text{-SMA}$ deposition in 7- and 28-day post-printing in the different support baths. Positive controls are arterial control for $\alpha\text{-SMA}$, ligament for type I collagen and articular cartilage for type II collagen. Quantification of immunofluorescence intensity normalised with background intensity and normalised to the number of nuclei of each region of interest (ROI) for $\alpha\text{-SMA}$ (B), collagen I (C), collagen II (D); $n=3$. For all the graphs, a two-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$.

Statistically comparable groups are labelled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

Figure 5.4. A stiffer support bath improves μ T fusion and the alignment of the secreted collagen fibres. A) Graphical abstract of a 4D bioprinting platform capable of generating phenotypically distinct musculoskeletal grafts. B) Histological assessment of bioprinted μ Ts after 28 days post-printing with picrosirius red staining, using PLM imaging to analyse collagen fibre alignment. Colour maps are generated from PLM images. Colour hue represents fibre orientation, with blue/cyan corresponding to fibres aligned at 0° and pink/red corresponding to fibres aligned at 90° . Sb: $200\mu\text{m}$. Quantification of the fibre orientation was then performed for the 0.25%, 0.5%, 1% and 2% XG-MA support baths. C) Average collagen fibre orientation with the filaments generated in the different support baths. D) Fibril diameter at 28 days post-printing. E) Fibre coherency was quantified, with values approaching 1 indicating highly aligned fibres and values closer to 0 reflecting greater dispersion in multiple directions ($n = 4$). F) Biochemical quantification of total collagen normalised to DNA levels. Statistical difference is determined using a two-way ANOVA test with Tukey's post hoc comparison test. Significance was accepted with $p < 0.05$. Statistically comparable groups are labelled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

Figure 5.5. The bioprinting of scaled-up anisotropic tissue grafts. A) Bioprinting of Benninghoff collagen arcades is typical of native AC. The collagen fibre orientation was quantified in the superficial (i), middle (ii) and deep (ii) regions of the tissue, indicating the development of highly anisotropic tissues. Coherency analysis values closer to 1 indicate that the fibres are aligned in one direction, while values close to 0 indicate the fibres are aligned in different directions. White indicates the colour map of the superficial zone, green indicates the colour map of the middle zone, and blue indicates the colour map of the deep zone. Scale bar: $200\mu\text{m}$. B) Bioprinting of circumferential filaments to engineer a meniscal tissue. Images from two different regions (green (i) and white (ii) boxes) are provided, again demonstrating that the collagen fibres are aligning according to the printing path. Fibre coherency analysis indicates that most of the fibres are aligned in one direction. The green lines/bars represent the collagen fibre orientation within the regions highlighted within the green boxes (i). The white lines/bars represent the collagen fibre orientation within the regions highlighted within the white box (ii). Scale bar: $200\mu\text{m}$. C) Bioprinting of a 3-layered anisotropic ligament tissue, where most of the fibres are aligned towards the 0° direction in both the lateral (yellow colour box (i)) and top (blue colour box (ii)) view. Scale bar: $200\mu\text{m}$. A one-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. *Indicates $p < 0.05$. ns: non-significant. For all the graphs: $n=3$. D) Histological and immunohistochemical assessment for the cartilage construct for sGAG, type II collagen, and type I collagen deposition compared to native AC. E) Histological and immuno-histological assessment for the meniscal construct for sGAG, type I collagen, and type II collagen, compared to the native menisci. F) Histological and

immunohistochemical assessment for the ligament construct for α -SMA, type I collagen, and type II collagen compared to the native ligament. Scale bars: 200 μ m for type I and II collagen and 100 μ m for α -SMA.

Figure 6.1. Rheological properties and cell viability of XG and XG-MA hydrogels.

(A) Viscosity of XG, XG-MA, and XG: XG-MA blends across shear rates of 0.1–10,000 s⁻¹, and zero-shear viscosity of the corresponding support baths. Group differences were assessed by one-way ANOVA with Tukey's multiple comparisons test; no significant differences were detected. (B) Swelling ratio (%) and (C) mass loss (%) for the selected blend ratios and XG-MA at day 0 (D0), day 14 (D14), and day 21 (D21). Swelling ratio was calculated as: $(W \text{ (mg) wet} - W \text{ (mg) dry}) / (W \text{ (mg) dry}) * 100$ at each time point. Mass loss was calculated as: $((W \text{ (mg) Day 0} - W \text{ (mg) Day X}) / W \text{ (mg) Day 0}) * 100$, where W Day 0 is the initial dry weight, and W Day X is the dry weight at the given point. Differences across groups and time were evaluated by two-way ANOVA with Tukey's post hoc test. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (D) Live/dead staining of printed μ Ts in the selected XG: XG-MA blends at day 28. Scale bar: 200 μ m.

Figure. 6.2. XG: XG-MA support the chondrogenic phenotype of bioprinted μ Ts at day 28 of culture in chondrogenic media.

A) histological staining for H&E, sGAG (AB), Collagen (PR) and Calcium deposit (AR) at day 28 of bioprinted μ Ts showing high sGAG and collagen deposition and negativity for calcium staining. B) biochemical analysis of bioprinted μ Ts at day 28 showing no significant differences between groups. Group differences were assessed by one-way ANOVA with Tukey's multiple comparisons test; no significant differences were detected.

Figure 6.3. Higher XG content in the support-bath formulation diminishes collagen alignment into a single preferred direction, reducing tissue anisotropy.

(A) Polarised light microscopy (PLM) of printed μ Ts at day 28 after Picrosirius Red staining, with corresponding fibre-orientation colourmaps. Colour hue encodes orientation: blue/cyan $\approx 0^\circ$ and pink/red $\approx 90^\circ$. Scale bar: 200 μ m. Immunofluorescence for specific collagen deposition at day 28, counterstained with DAPI. Scale bar: 150 μ m (B) Quantification of fibre orientation for each support bath. (C) Fibre coherency, where values near 1 indicate strong alignment and values near 0 indicate greater dispersion ($n = 3$). (D–E) Quantification of immunofluorescence signal for the support baths, normalised to the number of nuclei per region of interest (ROI) ($n = 3$). Group differences in panels C–E were assessed by one-way ANOVA with Tukey's multiple comparisons test; no significant differences were detected.

Figure 6.4. Incorporating XG at a 1:1 XG: XG-MA ratio promotes support-bath degradation and enhances inter-filament fusion in scaled-up tissue sheets.

(A) Brightfield micrographs of printed tissue sheets immediately post-printing (D0) and at day 7 (D7), showing progressive fusion of printed μ Ts. (B) Macroscopic photographs

of tissue sheets at D0 and day 28 (D28); the 1:1 XG: XG-MA blend exhibits improved fusion between filaments, yielding a more cohesive graft. Scale bar: 0.5 cm. (C) Histology at D28: H&E, sulphated GAGs (Alcian Blue, AB), calcium deposits (Alizarin Red, AR), and total collagen (Picrosirius Red, PR). (D) Biochemical analysis at D28 versus native meniscus: sGAG/DNA, collagen/DNA, %sGAG per wet weight, and sGAG/collagen. Group differences were evaluated by one-way ANOVA with Tukey's multiple comparisons test; significance was accepted at $p < 0.05$. * $p < 0.05$, ** $p < 0.01$.

Figure 6.5. XG: XG-MA support baths promote interfilament fusion while preserving anisotropy in scaled-up tissue sheets at day 28. (A) Polarised light microscopy of Picrosirius Red–stained tissue sheets at day 28 with corresponding fibre-orientation colour maps; hue encodes orientation (blue/cyan $\approx 0^\circ$, pink/red $\approx 90^\circ$). Insets show higher-magnification views of individual filaments, highlighting strong intra-filament anisotropy with matching colour maps. (B) Quantification of fibre-orientation distributions for each support bath. (C) Fibre coherency, where values approaching 1 indicate strong alignment and values near 0 indicate greater dispersion ($n = 3$).

Figure 6.6. Degradability of the XG: XG-MA support bath enables the fabrication of mechanically functional bioprinted meniscal tissues. (A) Schematic of the bioprinting design and resulting constructs: poor interfilament fusion yields tissues that remain anisotropic along the y-axis but are mechanically weak and poorly integrated along the x-axis, whereas improved interfilament fusion preserves anisotropy while increasing cohesion and Young's modulus along the x-axis. (B) Macroscopic images of the tensile testing setup with a displacement grid highlighting regions of highest (red) and lower (yellow) deformation. Scale bar: 3 mm. (C) Ramp modulus of printed tissue sheets in the x- and y-directions. (D) Equilibrium modulus of printed tissue sheets in the x- and y-directions. Statistical significance was assessed by two-way ANOVA followed by a post hoc multiple comparison test. Significance was accepted at $p < 0.05$. * $p < 0.05$; ** $p < 0.01$.

Figure 7.1. MSCs μ Ts show distinct patterns of growth and matrix deposition after 28 days in culture under different growth factor regimes. (A) Schematic of the growth factor regimens applied during μ T fusion. μ Ts were fused at day 2 post formation, and the different regimes were applied. At day 14, the growth factors were switched in regimes C and D. At day 28, the study was stopped and the samples analysed. Step 1: μ T fabrication; step 2: μ T fusion; step 3: differentiation of fused μ Ts. (B) Brightfield and histological assessment of fused μ Ts after 28 days under the following regimens: TGF- β 3 for 4 weeks; CTGF for 4 weeks; TGF- β 3 for 2 weeks followed by CTGF for 2 weeks; CTGF for 2 weeks followed by TGF- β 3 for 2 weeks; and combined TGF- β 3 + CTGF for 4 weeks. Histological stains: H&E (morphology), Alcian Blue (sGAG), Picrosirius Red (total collagen), and Alizarin Red (calcium deposit/mineralisation) (C) μ T diameter at day 7 (D7) and day 28 (D28). (D) Diameter fold change across regimens, defined as D28/D1. $N=3$, significant differences were determined

using a two ANOVA followed by a Tukey's test post hoc comparison for (C) and an ordinary one-way ANOVA followed by a Tukey's multiple comparison test for (D).

Figure 7.2. Self-assembled μ Ts show distinct patterns of growth and matrix deposition after 28 days in culture under different growth factor regimes. (A) DNA quantification revealed differences in cellularity, with the CTGF+TGF- β 3 group containing significantly more cells than CTGF alone. (B) sGAG accumulation was highest in groups exposed to TGF- β 3, particularly in the TGF- β 3 and CTGF+TGF- β 3 groups, whereas sequential treatments resulted in reduced deposition. (C) Collagen content followed a similar trend, with greater accumulation when TGF- β 3 was present during the early phase of culture, and a modest increase in the CTGF+TGF- β 3 group compared to TGF- β 3 alone. (D-E) When normalised to DNA, both sGAG and collagen deposition confirmed these differences, highlighting the importance of growth factor regime in regulating matrix biosynthesis. (F) The sGAG-to-collagen ratio further emphasised the distinct balance between cartilaginous and fibrous matrix production across groups. (G) Wet weight measurements showed comparable mass in TGF- β 3 and CTGF+TGF- β 3 groups, whereas CTGF constructs were significantly smaller. (H-I) Normalisation of matrix components to wet weight revealed that TGF- β 3 induced the highest sGAG percentage, while collagen percentage was greatest in the TGF- β 3 $\rightarrow$ CTGF and CTGF+TGF- β 3 groups, though values remained below native meniscus levels. N=4, significant differences were determined using an ordinary one-way ANOVA followed by a Tukey's multiple comparison test, where * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, and **** denotes $p < 0.0001$.

Figure 7.3. Microtissues develop a distinct pattern of ECM deposition when exposed to different growth factor regimes. μ Ts were harvested at 2 days post fabrication and fused in a low adherent agarose mould and kept in culture for 28 days at different growth factor regimes: 1) TGF- β 3 only for 4 weeks(TGF- β 3) 2)CTGF only for 4 weeks (CTGF) 3)TGF- β 3 for 2 weeks and switch to CTGF for the remaining two weeks (TGF- β 3 $\rightarrow$ CTGF) 4) CTGF for 2 weeks and subsequently TGF- β 3 for the remaining 2 weeks (CTGF $\rightarrow$ TGF- β 3) 5) CTGF and TGF- β 3 together for 4 weeks (CTGF+TGF- β 3). At the end of the culture, the resultant grafts were stained against chondrogenic markers, collagen type I (COL1) and collagen type II (COL2). Scale bar: 200 μ m.

Figure 7.4. Localised and sustained delivery of growth factors in bioprinted μ Ts affects tissue compaction and cell viability. A) Schematic representation of the bioink formulation and printing of μ Ts at a density of 45,000 μ Ts/mL into an XG-MA support bath. B) Viscosity profiles of gelatin bioink and laponite+ gelatin-based bioink measured over a shear rate range of 0–1000 s^{-1} at 4 $^{\circ}C$ (N = 3). C) Brightfield microscopy images of printed μ Ts in fibro-ink supplemented with different growth factors over 21 days of culture, showing tissue compaction and μ T fusion. D) Release of growth factors from the bioinks assessed by ELISA (N = 3). E) Filament diameter of printed μ Ts in fibro-ink across different conditions (N = 12). F) Viability assessment of printed μ Ts in fibro-ink after 28 days in culture, post-printing. Significant differences were determined using one-way

ANOVA followed by Tukey's multiple comparison test, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Figure 7.5. Printed μ Ts remodel their ECM toward a fibrocartilage phenotype within 28 days when encapsulated within a controlled release bioink. μ Ts were bioprinted into 1% XG-MA support bath and encapsulated into 1% gelatin and 0.05% LAP (w/v) supplemented with growth factors as follows: TGF- β 3 for 4 weeks, CTGF+TGF- β 3 for 4 weeks, or no growth factors in the ink but added in the medium (CDM+ group). A) Histological assessment of chondrogenic markers: H&E, Alcian Blue (sGAG), and Alizarin Red (calcium deposits). Scale bar: 120 μ m. B–G) Biochemical quantification: DNA (B), sGAG (C), total collagen (D), sGAG/DNA (E), collagen/DNA (F), and sGAG/collagen (G). Outliers were identified using a ROUT test. Data were analysed by one-way ANOVA followed by Tukey's post hoc test. Statistical significance was defined as $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

Figure 7.6. Printed μ Ts into laponite based bioinks exhibit distinct collagen alignment profiles under distinct growth factor regimes. A) Histological staining with Picrosirius Red/Alcian Blue to visualise both collagen and sGAG deposition over 28 days, alongside polarised light microscopy (PLM) and OrientationJ-derived colour maps. Colour hue indicates fibre orientation: blue/cyan = 0°, pink/red = 90°. Scale bar = 120 μ m. B) Average collagen fibre orientation under different growth factor regimes with laponite-mediated slow release. C) Fibre coherency, where values close to 1 represent highly aligned fibres and values near 0 represent dispersed orientations ($n = 3$). Scale bar = 200 μ m. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc test. Statistical significance was set at $p < 0.05$.

Figure. 7.7 Localised growth factor delivery alters collagen deposition and gene expression in printed μ Ts. A) Immunohistology staining for collagen I (green) and collagen II (red) in bioprinted μ Ts. Native inner and outer meniscus regions are shown for comparison, highlighting regional differences in collagen deposition. B qPCR analysis of bioprinted μ Ts with localised growth factor delivery, showing differential expression of chondrogenic and fibrochondrogenic markers, including collagen I (COL1), collagen II (COL2), COL1/COL2 ratio, aggrecan (ACAN), tenascin (TNC), and tenomodulin (TNMD). Data were analysed using one-way ANOVA, with significance accepted at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 7.8. Dual-cartridge bioprinting enables precise fabrication of scaled-up grafts and region-specific constructs for meniscal regeneration. (A) Schematic of the dual-cartridge bioprinting workflow: MSC-derived μ Ts are deposited within an XG-MA support bath using two bioinks, a chondro-ink (+TGF- β 3) and a fibro-ink (+CTGF + TGF- β 3), to recapitulate meniscal zonal composition. Zonal patterning strategy: the outer zone is printed with fibro-ink (CTGF + TGF- β 3) and the inner zone with chondro-ink (TGF- β 3). Macroscopic image of

three zonally defined bioprinted menisci immediately post-printing (day 0) in a 6-well plate. (B) Brightfield microscopy images of bioprinted filaments in the engineered inner and outer zones at day 0 and day 28; the dashed line marks the boundary between zones. Scale bar: 200 μm . (C) Histological evaluation of engineered inner and outer zones: H&E, Alcian Blue (sGAG), Picrosirius Red (collagen) and PLM. (D) Immunofluorescence for collagen I, collagen II, with DAPI for nuclei; native inner and outer meniscus shown for comparison.

Fig. S3.1. Single μT s show high viability when cultured for 2 days in CDM in a high-throughput microwell system. Viability assessment of the single μT s after 2 days in chondrogenic media cultured in 5 mL of CDM with a final density of 7.5×10^3 cells/mL. Most of the μT s were positive for live cells stained in green for Calcein-AM and dead cells stained in EthD-1. Only 2 samples are shown as representative images. Scale bar: 100 μm .

Figure S3.2. Trilineage differentiation of bone marrow-derived MSCs. (A) Adipogenic differentiation of MSC monolayers cultured for 21 days in adipogenic differentiation medium (ADM) or maintained in expansion medium (XPAN, control). Top: macroscopic images of 6-well plates showing triplicate wells per condition. Bottom: representative micrographs of Oil Red O-stained cultures at 4 $\times$, 10 $\times$, and 20 $\times$ magnification, highlighting intracellular lipid droplet accumulation in ADM-treated cells. (B) Osteogenic differentiation of MSC monolayers cultured for 21 days in osteogenic medium or XPAN (control). Top: macroscopic images of 6-well plates with triplicate wells per condition. Bottom: representative micrographs of Alizarin Red-stained cultures at 4 $\times$, 10 $\times$, and 20 $\times$ magnification, showing calcium-rich mineralised matrix in osteogenic cultures. (C) Chondrogenic differentiation in 3D pellet culture after 21 days in chondrogenic medium. Pellets were sectioned and stained with H&E (general morphology), Alcian Blue (sGAG-rich cartilage matrix), Picrosirius Red (collagen), and Alizarin Red (mineralisation). Scale bar = 100 μm .

Figure S3.3. Estimated output volume of fused μT constructs. Construct volumes were calculated from measured diameters in brightfield images assuming spherical geometry. Based on average construct diameters of ~ 1.8 mm and ~ 2.8 mm, the corresponding theoretical volumes were ~ 3.05 mm³ and ~ 11.49 mm³, respectively. Data are shown for constructs formed from 50, 150, and 300 μT s across different time points, with variability reflecting differences in construct size and morphology. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. Results are presented using a compact letter display due to multiple comparisons, where groups sharing the same letter are not significantly different.

Fig. S3.4. TUNEL staining for apoptosis. Representative images of the core of a scaled-up graft from caprine MSCs stained with a green nuclear stain and a red TUNEL stain. TUNEL-positive apoptotic cells are those in which the green and the red colocalise to the same cell. Scale bar: 200 μm .

Figure S4.1. Xanthan gum support bath does not favour chondrogenesis but fibrochondrogenesis and hypertrophy. DAB immunohistochemistry for collagen type I, II and X. Isotype controls included ligament sections with and without primary antibody for collagen type I; AC as a positive control for collagen type II and bone as a negative control for collagen type II; bone as a positive control for collagen type X; and AC as a negative control for collagen type X. Scale bar: 200 μm .

Fig. S4.2. Microtissue viability post-encapsulation with gelatin prior to bioprinting. Live/dead of μT s (2000 cells/ μT) encapsulated in gelatin 1% prior to printing show high viability of μT s. Green: Calcein (Live cells), Red: Ethidium Bromide (dead cells). Sb: 200 μm .

Fig. S5.1. Perfusion of black dye through a void channel formed after the diffusion of a printed 1% gelatin filament within the microenvironment. Following printing and UV curing of the support bath, the gelatin bioink liquefied and diffused into the surrounding microenvironment, leaving behind a void channel. This channel was subsequently perfused with black dye to confirm that the channel remained open, illustrating how gelatin diffusion supports the temporal reduction in filament diameter over time. Scale bar: 300 μm .

Fig. S5.2. Rheological properties of XG-MA at different concentrations and UV-curing depth on the bath stiffness. A) Viscosity dependency on shear rate of the support bath at different concentrations, from low shear rate (0.1 s^{-1}) to very high shear stress (1000 s^{-1}) demonstrating shear-thinning properties B) Shear stress dependency on shear rate of the support bath at different concentrations, from low (0.1 s^{-1}) to high (1000 s^{-1}) shear stress C) Stress-relaxation behaviour of the XG-MA support bath at different concentrations to assess the recovery of the material post applying high shear stress D) Graphical representation of mechanical testing of both halves (top and bottom) of the support bath post UV exposure to assess UV curing depth E) macroscopic pictures of acellular (1% gelatin only) printed filaments into support baths at different concentrations, with different optical properties F) mechanical testing of both halves (top and bottom) of each gel to assess curing depth dependency of all concentrations of the support bath post-UV exposure (4 min). A two-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. ns: non-significant.

Fig. S5.3. Quantification of filament smoothness. A) Brightfield microscopy images were used to assess local diameter variations of printed μT filaments. For each filament, five diameter measurements were taken at distinct regions along its length ($n = 3$ images per condition). The brightfield images represent the printed μT s

at day 0 under different conditions (refer to Fig. 1). B) The standard deviation of these measurements was calculated as an index of smoothness, with lower values indicating more uniform and smoother filaments, and higher values reflecting increased irregularity. This parameter helps assess μ T fusion and filament compaction post-printing. 2-way ANOVA followed by Tukey's post hoc comparison test was performed to assess the differences between the groups; significance was accepted when $p < 0.05$. Statistically comparable groups are labelled using compact letter display where groups sharing the same letter are not significantly different ($p > 0.05$).

Fig. S5.4. Stiffness of the supporting bath post printing influences the deposition of sGAGs and matrix re(modelling) over time in culture. A) Live/dead staining of printed μ Ts into XG-MA support bath, 7 days after printing. Green: calcein AM; Red: Ethidium Bromide. Sb: 100 μ m. B) Histological evaluation of the printed μ Ts after 28 days in culture with H&E, Safranin-O (SAF-O) and Alcian Blue to evaluate sGAGs deposition, which decreases with the increase in stiffness of the XG-MA support bath. Sb: 200 μ m. C) Biochemical quantification of total DNA, sGAG and collagen per printed filament. Statistical difference is determined using a two-way ANOVA test with Tukey's post hoc comparison test. Significance was accepted with $p < 0.05$. * indicates $p < 0.05$, **** indicates $p < 0.0001$.

Fig. S5.5. (Re)modelling of the collagen fibers along the printing direction after 28 days in culture. Scanning electron microscope (SEM) pictures of bioprinted μ Ts to show the ECM organization and (re)modelling, driving tissue maturation and differentiation. Sb: 10 μ m.

Fig. S5.6. The spacing (distance) between filaments post-printing modulates the anisotropy of a scaled-up bioprinted tissue construct. A) Brightfield microscope pictures of bioprinted μ Ts 28 days post printing, showing fusion of the μ Ts and adequate distance between each filament. B) Polarised Light Microscope images of bioprinted filaments at different distances after 28 days of culture in TGF- β 3-supplemented media, in a support bath with variable stiffness. C) The mean average of the collagen fibres indicates that the spacing of the filaments into a scaled-up construct influences the final anisotropy of the printed construct. D) Fibre coherency shows highly aligned tissues. Colour maps are generated from PLM images. Here, colour hue is used to indicate fibre orientation, where blue/cyan indicates fibres oriented at 0 degrees and pink/red indicates fibres oriented at 90 degrees. For all the graphs, a one-way ANOVA test was performed, followed by Tukey's post hoc comparison to compare the means of each group at different time points. Significance was accepted when $p < 0.05$. * Indicates $p < 0.05$. **Indicates $p < 0.01$. For all the graphs: $n = 3$.

Fig. S5.7. Macroscopic images of scaled-up tissue grafts. A) Cartilage construct B) Meniscus constructs C) Ligament tissue construct.