Integrating melt electrowriting and inkjet bioprinting for engineering structurally organized articular cartilage

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ABSTRACT

Successful cartilage engineering requires the generation of biological grafts mimicking the structure, composition and mechanical behaviour of the native tissue. Here melt electrowriting (MEW) was used to produce arrays of polymeric structures whose function was to orient the growth of cellular aggregates spontaneously generated within these structures, and to provide tensile reinforcement to the resulting tissues. Inkjet printing was used to deposit defined numbers of cells into MEW structures, which self-assembled into an organized array of spheroids within hours, ultimately generating a hybrid tissue that was hyaline-like in composition. Structurally, the engineered cartilage mimicked the histotypical organization observed in skeletally immature synovial joints. This biofabrication framework was then used to generate scaled-up (50 mm × 50 mm) cartilage implants containing over 3,500 cellular aggregates in under 15 min. After 8 weeks in culture, a 50-fold increase in the nonlinearity mimetic of the native tissue. Helium ion microscopy further demonstrated the development of an arcading collagen network within the engineered tissue. This hybrid bioprinting strategy provides a versatile and scalable approach to engineer cartilage biomimetic grafts for biological joint resurfacing.

1. Introduction

Under normal physiological conditions, articular cartilage is capable of transmitting loads of several times body weight through synovial joints for decades [1]. However due to its avascular nature and relatively limited regenerative capacity, injury to articular cartilage can progress leading to further degeneration of the joint [2]. Osteoarthritis (OA) is characterized by progressive loss of articular cartilage tissue and function and is a debilitating disease affecting millions of people worldwide [3,4]. For patients suffering from end-stage OA, total joint replacement is the standard surgical treatment to restore mobility. While this procedure is well established, it does not provide a long-term solution and is a debilitating disease affecting millions of people worldwide [5]).

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differences, tissues engineered via such self-assembly processes produce a more hyaline-like matrix compared to standard scaffold or hydrogel encapsulation \[10,11\], and can generate cartilage tissues with biochemical and biomechanical properties within the range of native articular cartilage \[12–14\]. It has also been shown that radial confinement increases collagen organization within self-assembled cartilage \[15\]. This is of particular interest since recapitulating the complex zonal organization of articular cartilage remains an important challenge \[16\], as this structure is integral to the ability of the tissue to withstand the challenging mechanical loading of synovial joints \[17–19\]. To further improve the structural organization of engineered cartilage tissues, hybrid approaches that combine the benefits of cellular self-assembly or self-organization with biofabrication techniques such as 3D bioprinting have recently been developed. Printed polymeric structures can be used to guide the deposition of a cartilage biomimetic collagen network within engineered tissue \[20\]. To date, polymeric structures with relatively thick fibers generated by fused deposition modeling (FDM) have been used to trigger cellular self-assembly and to direct subsequent tissue organization \[20,21\]. These large polymeric fibers lead to the development of overly stiff and non-compliant constructs \[22\] that are not mimetic of the native tissue and could potentially damage opposing joint surfaces if implanted in vivo. Ideally, polymeric reinforcement within such hybrid engineered tissues would mimic the functionality provided by the collagen network in articular cartilage, specifically a fibrillar matrix that primarily sustains tensile loads \[19\], but in isolation contributes little to the compressive properties of the tissue \[23\].

Melt electrowriting (MEW) uses voltage-stabilized jets to accurately place low micrometer scale fibers in pre-defined locations in 3D space \[24\]. The fiber diameter ranges from 820 nm \[25\] to 140 μm \[26\]; whereas it is usually over 200 μm with FDM, limiting the capacity of this additive manufacturing technology to produce truly biomimetic implants \[27,28\]. In contrast, MEW enables the development of highly porous (80–98 vol% pore volume), sophisticated and biomimetic scaffolds \[29,30\]. For example, MEW has been used to mimic the anisotropy of the collagen network in cartilage \[31,32\], mechanically reinforcing hydrogels in a way that recapitulates the behaviour of collagens in cartilage \[31\]. MEW scaffolds have also been used as a substrate for the assembly of pre-formed multicellular spheroids \[33,34\]. Here, we hypothesized that a MEW network could guide cartilage-specific tissue organization during the growth of self-assembled cell aggregates, while simultaneously reinforcing the resulting hybrid construct in a manner analogous to that of the collagen network in articular cartilage, specifically providing tensile strength and stiffness but contributing little to the compressive properties of the tissue in the absence of proteoglycans.

To this end, we combined MEW and inkjet bioprinting into a sequential biofabrication framework where a defined number of mesenchymal stem cells (MSCs) were ink-jetted into box-like MEW scaffolds, which supported spontaneous cellular aggregation within each microwell (Fig. 1). We demonstrate how the association of such additive manufacturing technologies can be used to produce sheet-like tissue constructs composed of multicellular spheroids maturing into stratified cartilage tissue, within only a very limited fraction of synthetic polymer (<2%). By expanding the capabilities of this novel multiple-tool biofabrication strategy, we also demonstrate that it can be used to engineer large and functional cartilage grafts with potential applications in biological joint resurfacing.

2. Results

2.1. Integrating melt electrowriting and inkjet printing to generate arrays of cellular spheroids

First, we developed a strategy to trigger the self-assembly of cellular aggregates from cells ink-jetted into micron-sized polymeric microchamber systems (which can also be referred to as pores) (Fig. 1). To this end, melted poly (ε-caprolactone) (PCL) was extruded across an electric field to produce an orthogonal array of microfibers (≈7 μm diameter) (Supplemental Table 1) (Supplemental Figure 1A-D). The microchamber height (≈0.75 mm) and spacing (≈0.8 mm) were kept constant throughout, resulting in a microchamber volume of 0.48 mm³. Subsequently, the MEW scaffold was placed onto a non-cell adhesive dish coated with poly (2-hydroxyethyl methacrylate) (pHEMA) that defined the temporary bottom boundaries and supported cell-aggregation post inkjetting. Lastly, the printed microchambers were loaded with cells by inkjet printing a cell suspension into each microchamber. The valve opening time, which defines the volume of one drop printed through a single valve opening, was kept constant and reproducible volumes were printed throughout the experiments (Fig. 2A). After identifying the volume corresponding to a single drop/valve opening (1.16 ± 0.06 μl), the concentration of the cell suspension was defined (30 million cells/ml) and inkjet printing was used to seed a defined number of cells (suspended in cell culture medium) into the confining hydrophobic

![Fig. 1. Biofabrication process. A box-like structure made of fibers in the micron-size range is produced by extruding poly (ε-caprolactone) (PCL) across an electric field (Melt Electrowriting or MEW). The MEW scaffold is centered in a plastic dish coated with a solution preventing cell adhesion to the dish (System Assembly). A droplet containing a defined number of cells is then printed in every single chamber of the scaffold through a piezoelectric valve (Inkjet Bioprinting). As cell adhesion is limited at the bottom of the assembly by the hydrophobic coating, and on the sides by the hydrophobic polymer, the cells aggregate and self-assemble into spheroids within the MEW scaffolds within 48H (Spheroid Self-Assembling).](image-url)
polymeric microchambers (34,845 ± 1,744 cells per microwell) (Fig. 2B).

Microscopic observations made immediately after printing showed a homogeneous cell suspension segmented by protruding MEW fibers, indicating individual filling of the microchambers with cells (Fig. 2C). A contracting cell layer was observed in each microchamber a few hours later (Fig. 2D), further condensing with time (Fig. 2E), resulting in a structured array of cellular spheroids within the MEW template 48 h later (Fig. 2F). Interestingly, spheroids self-assembled predominantly in the corner of the microchambers and against each other, exhibiting a regular pattern. Temporal monitoring of the self-assembly process showed that a critical point is reached during the contraction phase where the spheroid detaches almost totally from the scaffold, keeping just one or two points of attachment, resulting in the spheroid shifting to a specific corner of the microchamber (Supplemental Video 1). Closer examination confirmed spheroids nesting at the bottom of the polymeric chambers (Fig. 3A, B), with signs of attachment to the microfibers (Fig. 3B-D) and cellular extensions protruding through the fiber walls (Fig. 3D). This last observation suggests that the spheroids were able to communicate physically through the fiber wall, facilitating their self-assembly into a regular pattern and eventual fusion. Finally, the spheroid diameter (266 ± 37 μm) was shown to be normally distributed (Fig. 2G), indicating that spheroids of reproducible size can be generated in every single microchamber of the MEW scaffold. Taken together, these results demonstrate that cellular condensation occurred following deposition of the cell suspension into microwells and that structured arrays of cellular spheroids can be engineered by integrating inkjet printing and MEW.

2.2. Self-organization of hyaline-like cartilage in MEW scaffolds following inkjet bioprinting

The structured array of cellular spheroids formed within the PCL template grew over time to fill the microchambers, with fusion between adjacent spheroids evident after 21 days macroscopically and in live/dead imaging (Fig. 4). The isolated spheroids observed at day 0 grew out of their chambers to fuse with their neighbours (Fig. 4C) and covered the surface with viable cells (Fig. 4E, F). Furthermore, microfiber walls clearly visible at day 0 and in empty scaffolds (Fig. 4B) could not be distinguished on hybrid tissue cross-sections that show a continuous tissue with a glossy appearance similar to native cartilage. Histological staining for sGAG deposition confirmed robust cartilage development and the formation of a highly connected material (Fig. 5A), with the sGAG content of the engineered tissues (2.5 ± 1.2 ww%) approaching
that of native articular cartilage (~2–10 ww% \cite{18,35}) (Fig. 5B). Although total collagen content of engineered tissues (0.9 ± 0.2 ww%) was an order of magnitude below that of articular cartilage (~5–30 ww% \cite{18,35}), hybrid tissues were hyaline-like in composition as evidenced by strong positive staining for type II collagen, while type I collagen was barely detected (Fig. 5C).

### 2.3. Engineered cartilage within MEW scaffolds mimics the spatial organization of articular cartilage from skeletally maturing joints

Microscopic observations of hybrid tissue sections stained for hematoxylin-eosin revealed cells with round morphology typical of chondrocytes, which were randomly distributed within the tissue but flattening parallel to the surface in the superficial layers (Fig. 6A). A similar cell organization is found in developing cartilage and is known to be associated with an organized collagen network. Therefore we next used polarized light microscopy (PLM) to determine the degree of organization of collagen fibrils in the engineered tissues, and compared them to articular cartilage from skeletally immature joints (Fig. 6B-E).

Tissue sections from native cartilage displayed multi-zonal features typical of maturing cartilage \cite{18,36,37} (Fig. 6B). A greenish mildly birefringent radial zone with collagen fibrils oriented perpendicular to the surface is sandwiched between a tangential zone at the surface and an isotropic zone underneath (where collagen fiber reorganization occurs), both showing a strong yellow birefringence with collagen fiber pattern-oriented parallel to the surface. A very similar collagen architecture was found in the engineered tissues, with a 3 layers organization matching that found in maturing tissue. The thickness of the radial zone of the native and engineered tissues was similar, as was the architecture of the collagen fibrils under polarized light (oriented at ~ 80–70°). The average fibril orientation and dispersion were then assessed in the tangential, radial, and isotropic zones of the tissues. Average fiber orientation was 4.55 ± 4.61°, 80.97 ± 7.36° and 14.49 ± 13.86° in the engineered tissue and 1.5 ± 1.2°, 85.89 ± 3.35° and 5.17 ± 4.96° in native maturing cartilage (Fig. 6C). Similarly, fiber dispersion was 18.71 ± 6.44°, 16.82 ± 6.47° and 19.31 ± 10.12° in engineered tissue and 8.49 ± 2.76°, 16.84 ± 2.42° and 16.01 ± 1.93° in native cartilage (Fig. 6D).

Statistical analyzes revealed little differences between the engineered and native tissues, highlighting the similarity in their collagen organizations. Coherency was also used as a measure of the anisotropy, tending to 1 if there is a dominant direction in the average region. Coherency differed only between radial zones, where a higher degree of organization was found in the engineered tissue (0.5 ± 0.09) compared to maturing native cartilage (0.24 ± 0.07) (Fig. 6E). Taken together, these analyzes demonstrate spatial changes in cell morphology and the organization of collagen fibrils through the depth of the engineered tissue that mimicked that seen in the articular cartilage of skeletally immature joints.

### 2.4. Scaled-up engineered cartilage possesses mechanical properties approaching that of native articular cartilage

We next explored whether our multi-tool biofabrication strategy could be used to produce implants of a size suitable for resurfacing complex articular surfaces such as the hip joint (Supplemental Figure 1. E-F). To do this, 60 × 60 mm MEW scaffolds were printed and maintained in a pHEMA coated 60 mm Petri dish with a moulded PCL holder so that the inkjet area was a 50 × 50 mm square. Cells were inkjet printed in the 3,591 microchambers in this region in about 15 min without interruption. A cell spheroid was obtained in every microchamber where cells were ink-jetted, with microtissues developing and merging only into the ink-jetted area over 8 weeks and leaving the edge of the scaffold empty (Fig. 7A and B). To evaluate if the engineered hybrid tissue was functional, a combined stress-relaxation and dynamic unconfined compression protocol was used to determine the mechanical properties of the tissue. Tissue constructs stress-relaxed and strain-stiffened similarly to articular cartilage \cite{38,39} (Fig. 7C). The compressive modulus of engineered tissues was 177 ± 30 KPa and 398 ± 55 KPa at 20% and 30% strain respectively (Fig. 7D), which represents a 50–76 fold increase compared to MEW scaffolds (4 ± 2 KPa and 5 ± 3 KPa) and approached native tissue properties (0.24–1.4 MPa \cite{39–41}). Equilibrium and dynamic modulus are two other important parameters used to quantify the mechanical function of engineered cartilage tissues \cite{42}. The equilibrium modulus is a measure of the compressive stiffness of the tissue solid matrix since it is recorded after the ramp and hold phase when fluid is no longer moving through the tissue. The
The equilibrium modulus of engineered composite tissues was 180 ± 13 kPa and 214 ± 24 kPa at 20% and 30% strain respectively (Fig. 7E), which is close to that of native tissue (0.2–2 MPa [18,39,43–46]) and represents a 20–27 fold increase compared to the empty MEW scaffolds (9 ± 2 kPa and 8 ± 2 kPa). Lastly, during the dynamic phase of the test, a cyclic displacement is applied to the tissue to test its capacity to generate fluid pressurization/load support, which is related to the permeability of the solid matrix. Dynamic moduli recorded for tissue constructs were 1.4 ± 0.2 MPa at 20% strain and 2.6 ± 0.3 MPa at 30% strain (Fig. 7F). These values are of a similar order of magnitude as the native tissue (5–60 MPa [39,47–51]) and represent a marked 26–51 fold increase compared to empty scaffolds (56 ± 18 kPa and 51 ± 23 kPa).

The tensile properties of the larger, scaled-up tissue-engineered cartilage composites were also investigated (Fig. 8C-G). Scaffolds were successively stretched and relaxed at 3, 6, and 9% strain, then ramped beyond the yield point (Fig. 8D). The overlapping of the stress-strain curves first suggested minimal differences in tensile properties between engineered cartilage and the empty MEW scaffolds. At 9% strain, tensile Young’s modulus was 1.4 ± 0.3 MPa and 0.9 ± 0.2 MPa (Fig. 8E), and equilibrium modulus was 1.7 ± 0.1 MPa and 1.5 ± 0.1 MPa (Fig. 8F) for the engineered tissues and empty scaffolds respectively. These values approach that of native cartilage (5–12 MPa for tensile Young’s modulus [41] and 5–25 MPa for equilibrium modulus [43,52,53]), but no significant differences were found between composite tissues and empty scaffolds. However, only the engineered cartilage composite displayed a strain-stiffening behaviour. Although it remains significantly lower than that of native tissue (0.8–25 MPa [54]), higher yield stress was recorded for engineered tissues (132 ± 29 kPa) compared to MEW scaffolds (96 ± 14 kPa), pointing to some reinforcement in tensile properties after tissue maturation. Taken together, these results demonstrate that upscaling the hybrid biofabrication process results in functional analog tissues that could potentially be used for joint resurfacing.

Lastly, scanning helium ion microscopy (SHIM) was used to visualize in more detail the spatial organization of the collagen fibrils in the engineered tissue (Fig. 9). Clear spatial changes in structural organization with depth were observed (Fig. 9, B1 and B2). Collagen fibrils had a predominantly parallel orientation at the surface of the tissue (Fig. 9, C1, F1, and C2, F2), and arcaded (Fig. 9, D1, G1, and D2, G2) to a perpendicular orientation more deeply (Fig. 9, E1, H1, and E2, H2), thus resulting in a Benninghoff-like architecture. Small fibrils (38 ± 12 nm in diameter) with abundant fibrillar connections were observed through the tissue, similar to maturing articular cartilage. The collagen network was denser toward the surface of the tissue with packed fibrils,
a feature observed in the native tissue [18]. These findings demonstrate that the hybrid biofabrication process can generate tissue analogs with spatial changes in collagen fibril orientation mimicking that of native cartilage.

3. Discussion

Hybrid biofabrication processes can leverage the specific advantages of different additive manufacturing (AM) technologies [30], creating new and otherwise inaccessible opportunities in the field of tissue engineering. Here we hypothesized that the process of cartilage self-assembly could benefit from the association of MEW and inkjet bioprinting. To this end, the objective was to jet cells into the individual chambers of supporting MEW scaffolds with the goal of driving cellular condensation and directing cartilage-specific tissue organization. We demonstrated that the combination of MEW and inkjet printing supports the self-assembly of organized arrays of mesenchymal aggregates that fused to form a highly connected tissue with sGAG content approaching that of native cartilage. The polymeric chambers were able to drive an articular cartilage-like histotypical organization within the hybrid tissue; specifically, the cell and collagen fibrils organization were found to match that of skeletal immature joints. It was also possible to integrate inkjet printing and MEW to engineer clinically sized cartilage grafts with biomechanical properties close to that of native cartilage. Taken together, these results demonstrate how the integration of different 3D printing techniques can make it possible to produce functional stratified cartilage tissues with low-polymer content (<2%) for biological joint resurfacing applications.

The combination of MEW PCL fibers (which are reasonably hydrophobic) and non-adhesive coating provided an environment that supported cellular condensation within each microchamber, interactions between cell-aggregates and their functional development. In a previous study, we used FDM to print a polymeric framework on a non-porous PCL base where spheroids self-assembled and produced robust stratified cartilage [20]. However, the piling of thick PCL fibers prevents cell-cell communication, whereas the stacking of small MEW fibers with random-opened frames in the fiber wall (due to more fibers at the interconnections than on the wall itself) offers multiple opportunities for aggregates to physically connect, even at an early stage, and generate highly dense tissues. In addition, the use of non-adhesive coating as a temporary base offers the possibility to easily remove the engineered tissue from the culture insert and use it as a patch for joint resurfacing, which would not be possible with a solid base fused to the microchamber system. The highly compliant nature of the MEW sheets also allows them to be fitted over complex surfaces post-printing (Supplementary Figure 1.F), which would not be possible using more rigid polymeric structures generated using AM techniques such as FDM.

Scanning electron microscopy (SEM) and macroscopic pictures show that cells were initially localised in the interior of the microchambers, and the resulting aggregates grew over time in culture and fused with adjacent spheroids through and over the surface of the microchambers. The engineered tissues had an sGAG content approaching that observed in native cartilage after only 21 days in culture [18]. Hybrid constructs were also hyaline-like in composition, staining strongly for type II collagen and weakly for type I collagen. While total collagen content at this timepoint was less than that of native tissue, the organization of the collagen network mimicked that of articular cartilage taken from 12 weeks old porcine synovial joints, with a three-layer organization typical of juvenile cartilage [55,56]. It should be noted that the color and intensity of collagen birefringence are influenced by the alignment of the collagen fibrils, the packing density [57], the diameter of the fibrils [58], and the presence of proteoglycans in the tissue section [59]. Hence, densely packed and highly aligned collagen fibrils of the tangential and isotropic zones display a yellow birefringence, whereas the loosely woven and developing radial zone is seen with a green birefringence, which will turn yellow as fibril diameter and alignment

![Figure 1](image1.png)
increase over time, as is seen during normal tissue maturation [18,55]. This is well supported by SHIM observations revealing a stratified collagen network composed of thin collagen fibrils, becoming denser toward the surface of the tissue. The fact that cells can reorganize extracellular matrix (ECM) components during collective migration is well known [30], and it has been shown that PCL microfibers can guide the growth of collagenous tissues in vivo [60]. Furthermore, it is also known that achieving some level of structural organization in the collagen network is possible by spatial-confining self-assembling tissues [15]. Therefore, this structured organization observed in hybrid tissues is likely due to the orientated growth enforced by the boundaries of the MEW fibers. This anisotropic organization will help to maintain load-bearing abilities similar to the native tissue [62], together with the developing collagen network continuing to mature following implantation.

The utility of the newly developed biofabrication process was further demonstrated by generating self-assembled spheroids in a scaled-up version of this article.)

While the compressive mechanical properties of the hybrid tissue were dramatically higher than that of empty control, the MEW network still dominated the tensile properties of the construct after 8 weeks of in vivo maturation. Previous studies have shown that the collagen matrix buckles under compressive strains in proteoglycan-depleted cartilage [23], pointing to its primarily role in providing tensile strength and stiffness in the tissue [19]. Indeed the compressive modulus of such proteoglycan-depleted cartilage can be as low as 3 kPa, which is comparable to our empty MEW scaffolds. To the best of our knowledge, no tissue engineering strategy has to date been able to generate constructs with native-tissue like levels of collagen content and organization. We believe that the relatively low collagen content observed in our engineered tissues, coupled with a collagen network organization more...
Fig. 7. Integrating melt electrowriting and inkjet for engineering large cartilage graft. MEW scaffolds of 60 × 60 mm were printed and bone marrow-derived MSCs were ink-jetted into all microchambers covering a square surface of 50 × 50 mm, then cultured for 8 weeks in chondrogenic media. Two scaffolds were produced. (A) Large MEW scaffold showing complete filling of the ink-jetted area with self-assembled spheroids. The scaffold is handled above the lid of a 60 mm cell culture petri dish demonstrating ease of handling. (B) Five-millimeter diameter cylindrical construct punched out of the main scaffold for unconfined compression testing. (C) Representative stress-time curve of tissue-engineered cartilage (yellow line) and empty MEW scaffold (blue line) highlighting the different steps of the unconfined compression testing procedure. (D) Compressive (or ramp) modulus, (E) equilibrium modulus, and (F) dynamic modulus in unconfined compression of engineered tissue (yellow bars) and empty scaffold (blue bars) when applying increasing levels of strain amplitude. The values given in the dot plots represent punched-out regions as data points, mean as a bar, and standard deviation as error bars. ** and *** indicate statistically significant differences (**p < 0.01, ***p < 0.001), whereas “ns” denotes no significance (one-way ANOVA). Dotted lines indicate the minimal value recorded for articular cartilage in literature. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 8. Uniaxial stress-relaxation tensile testing of large engineered tissues. (A, B) Histological cross-section of engineered tissue stained for safranin-O and observed at (A) very low and (B) low magnification. (C) Pictures of a 20 × 5 mm tissue section sampled from the engineered tissue at the start and the end of the tensile testing procedure. (D) Representative stress-strain curve of tissue-engineered cartilage (yellow line) and empty MEW scaffold (blue line). Dotted lines indicate peak strain at 3, 6, and 9% strain that were each followed by relaxation before increasing strain amplitude. (E) Ramp and (F) equilibrium tensile modulus as well as (G) yield strength in uniaxial stress-relaxation tensile testing when applying increasing levels of strain amplitude on engineered tissue (yellow bars) or empty scaffold (blue bars). The values given in the dot plots represent test sections sampled from the engineered tissues or empty scaffolds as data points, mean as a bar, and standard deviation as error bars. *, ** and *** indicate statistically significant differences (*p < 0.05, **p < 0.01 and ***p < 0.001, one-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
mimetic of juvenile articular cartilage than fully mature cartilage, could explain the finding that the matrix deposited within the MEW network did not significantly increase the overall tensile modulus of the graft. However, in vitro maturation did increase the tensile yield strength of the construct, and the tensile ramp modulus of the hybrid tissue was still in the MPa range. It is anticipated that the continued maturation and reinforcement of the collagen network after implantation will improve the tensile properties, as is observed during postnatal development [63]. Lastly, the tensile modulus of the hybrid tissue was higher than the compressive modulus (tension-compression non-linearity), which is typical of articular cartilage mechanical properties and plays a fundamental role in its ability to support physiological levels of stress [48,64].

Our results suggest that the small PCL fibers printed by MEW played a key role in achieving non-linear tension-compression behaviour by directing the growth and maturation of self-assembled MSCs aggregates into cartilage tissue and secondly by providing tensile reinforcement.

Tissue engineering scalable cartilage grafts requires satisfying the physio-chemical demands of large volumes of cells, which can lead to inhomogeneous deposition of cartilage matrix if nutrient and oxygen are not sufficiently provided during in vitro maturation [65–67]. These considerations become exacerbated with scaffold-free approaches which require a high cell-seeding density to engineer even small tissues. Here, the culture of hybrid tissues was performed in static conditions, which may have led to areas with

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**Fig. 9. High-resolution visualization of collagen fibril orientation.** The upper panel (A, B1–H1) shows scanning helium ion microscopy images of the collagen network within engineered tissue cultured for 8 weeks in chondrogenic media. High-resolution images were acquired at the surface of the sample cross-section to observe the shift in fibril orientation. (B2–H2) Images were post-processed with the orientationJ plugin in ImageJ to build a color map of fibril orientation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
insufficient nutrients for spheroid maturation and fusion, and hence tissues with non-anatomically relevant thickness. Introducing dynamic bioreactor culture should address these points and increase the overall mechanical properties and thickness of the engineered grafts [20]. Future work is also required to evaluate if the hybrid tissues can integrate, sustain relevant mechanical loading, and further mature in vivo. Although we hypothesized that this new biofabrication strategy could be used to resurface complex joints, we did not address this challenge. Future work will look into the possibility to combine inkjet bioprinting with anatomically relevant scaffolds printed by MEW [29] for complex joint resurfacing. For example, microchambers could be printed directly onto a curved surface [68], enabling stratified cartilage to be engineered onto complex orthopedic implants such as a hip resurfacing implant.

### 4. Conclusion

In this experimental work, we combined different AM technologies (MEW, inkjet) to engineer stratified cartilage tissues. The majority of bioprinting approaches developed to engineer cartilage have used overly-stiff and non-compliant structures to reinforce hybrid tissues, which does not mimic the role of the collagen network in articular cartilage. In addition, recapitulating the stratified zonal architecture of this tissue is a major challenge in the field. We have addressed both of these challenges by generating arrays of spheroids within a MEW polymeric framework that orientated the growth and organization of the ECM secreted by the cells. This resulted in scalable tissues with a spatial collagen fiber organization mimicking that of skeletally immature joints and exhibiting tension-compression non-linear behaviour. Overall, this new hybrid biofabrication approach can create more biomimetic tissues compared to existing tissue engineering strategies, and could potentially be used to produce alternative treatment options for damaged or diseased articular cartilage.

### 5. Materials and methods

#### 5.1. Cell isolation and expansion

Bone marrow-derived MSCs were isolated from the femoral shaft of a porcine donor (Danish Duroc, male, 4 months old). 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 μg/ml) (all from Biowest) 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 resuspended in fresh expansion medium twice before it was filtered through a 40 μm 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 × 10^5 cells/cm² at 5% P02 in expansion medium supplemented with 5 ng/ml of fibroblast growth factor (FGF)-2 (PeproTech Ltd). Medium change was performed three times per week.

#### 5.2. Biofabrication process (MEW, pHEMA coating and inkjet-bioprinting)

All constructs were printed with the 3D Discovery multi-head printing system (RegenHu, Switzerland). MEW was performed with PCL (Capa® 6500D, Perstorp UK Ltd) molten in a metallic cartridge at 140 °C. PCL was extruded through a 24G nozzle with an air pressure of 0.056–0.08 MPa and voltage of 10 kV. The printhead was kept at a constant Z-coordinate of 3 mm and translated at a speed of 40 mm/s in X and Y directions over a fixed collector plate. The MEW jet was stabilized before printing by printing 8 lines which were analyzed for deviations in fiber diameter and/or pulsing. 60 × 60 mm box-like structures composed of 200 layers were printed. Each fibrous layer was orientated at 90° to the previous layer with 0.8 mm spacing between fibers. Accordingly, the walls of the microchambers consisted of 100 stacked fibers. The scaffolds were subsequently cut into 8 × 8 mm squares with a scalpel or directly sterilized with ethylene oxide.

To prevent cell adhesion, 12 well plates or 60 mm Petri dishes (Corning) were coated with 1.2% (w/v) pHEMA (Sigma-Aldrich, ref. P3932) at a density of 70 μl/cm² as previously described [69]. Sterile MEW scaffolds were placed onto the pHEMA coating and kept in place with a custom made metal or PCL ring so that the inkjet area was a 6 × 6 mm or a 50 × 50 mm square. Scaffolds were carefully washed with 1x Phosphate Buffered Saline (PBS) solution 3 × 5 min to set connections between the two hydrophilic materials (pHEMA coating and PCL) and to prevent the scaffolds from being resuspended when culture medium was added after cells were inkjet.

For inkjet bioprinting, a piezoelectric valve with an inner diameter of 0.3 mm and a stroke of 0.1 mm (Fritz Gyger AG, Switzerland, ref. 00015,815) was attached to a contactless dispensing printhead. The printhead was aligned with the centre of a single microchamber and was kept at a constant Z-coordinate of 40 mm. Next, the printhead was translated according to an alternating horizontal path at a speed of 4 mm/s. The piezoelectric valve opened for 1300 μsec every 0.8 mm to inkjet cells resuspended in expansion medium at a density of 30 × 10^6 cells/ml with an air pressure of 0.1 MPa. Post-printing, constructs were placed for 10 min in the incubator to initiate cell aggregation before adding excess expansion medium to the construct.

#### 5.3. Chondrogenic conversion

Chondrogenic medium was added to the constructs 48 h after inkjet bioprinting and consisted of hgDMEM supplemented with penicillin (100 U/ml) – streptomycin (100 μg/ml), 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbic acid-2-phosphate, 4.7 μg/ml linoelic acid, 1.5 mg/ml bovine serum albumin (BSA), 1 X insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma-Aldrich), and 10 ng/ml human transforming growth factor-beta (TGF-b) 3 (PeproTech Ltd). Cells were cultured at 5% P02 for at least 21 days and up to 8 weeks with medium change performed every two days.

#### 5.4. Scaffold imaging and spheroid measurement

Cell-seeded scaffolds were imaged with an Olympus SZX5 stereo-microscope and an Olympus IX71 optical microscope. To measure the size of spheroids, the freehand selection tool in ImageJ software (National Institutes of Health, USA) was used to measure the area of the cell aggregate. The diameter of a circle of equal projection area (Da) was then calculated using the equation $Da = 2\sqrt{A/x}$ where A is the area measured [70].

#### 5.5. Scanning electron microscopy

Samples were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (all from Sigma-Aldrich) at 4 °C for a minimum of 12 h. They were then rinsed twice in 0.1 M cacodylate buffer for 10 min, dehydrated in graded ethanol baths series, immersed twice in hexamethyldisilazane (Sigma-Aldrich) for 30 min and allowed to completely dry overnight before imaging. Samples were imaged with a Zeiss ULTRA plus scanning electron microscope and images colored with GIMP software (version 2.10.22).

#### 5.6. Live/dead confocal microscopy

Cell viability was assessed using a Live/Dead assay kit (Biosciences). Tissue constructs were rinsed with PBS and incubated in PBS containing 4 μM ethidium homodimer-1 and 2 μM calcein for 30 min. Samples were rinsed again in PBS and imaged with a Leica SP8 scanning confocal
microscope at 515 and 615 nm channels. Images were analyzed using Leica Application Suite X (LAS X). All images presented are 3D Z-stack reconstructions of the tissue. The depth reconstruction images were produced with Imaris software (BITPLANE, Oxford Instruments).

5.7. Time-lapse cell imaging

The self-assembling of spheroids was imaged with a CytoSMART™ Lux2 system (CytoSMART technologies, Netherlands).

5.8. Biochemical analyzes

After 21 days of in vitro culture constructs were washed in PBS, weighed, and frozen for subsequent analyzes. Each construct was digested with papain (3.88 units/ml) in 100 mM sodium phosphate - 5 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 6.5) with 10 mM l-cysteine-hydrochloride (all from Sigma-Aldrich) at 60 °C and 10 rpm for 18 h. DNA content was quantified using the Hoechst Bisbenzimide 33,340 dye assay, with a cell thymus DNA standard. The amount of sulphated glycosaminoglycan (sGAG) was quantified using the dimethylmethylene blue dye-binding assay (DMMB) (Blyscan, Blyscan Ltd.), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content using the dimethylmethylene blue dye-binding assay (p modifications).

Collagen types I and II were also evaluated using a standard immunohistochemical technique. Rehydrated sections were treated with pronase (32 PUK/ml, Sigma-Aldrich) at 37 °C for 5 min, then incubated in blocking buffer containing 1% (w/v) BSA and 10% (v/v) goat serum (all from Sigma-Aldrich) in 1X PBS for 1 h at room temperature (RT) to block non-specific sites. Tissue sections were then incubated with type I collagen (Abcam, ref. 90,395, mouse monoclonal IgG, 1:400) or type II collagen (Santa-Cruz, ref. sc-52658, mouse monoclonal IgG, 1:200) primary antibody diluted in blocking buffer overnight at 4 °C in a humidified chamber. Samples were then incubated with 3% (v/v) hydrogen peroxide solution (Sigma-Aldrich) for 20 min to block endoperoxidase activity, then with secondary antibody (Sigma-Aldrich, ref. B7151, anti-Mouse IgG) diluted in blocking solution (1:200 for detection of type I collagen and 1:300 for detection of type II collagen) for 1 h at RT. Following a 45 min incubation period with ABC reagent (ABC Elite kit Vectastain PK-400, Vector Labs), the DAB substrate (SK-4100, Vector Labs) was added to the tissue section and the presence of the protein of interest was revealed by the apparition of brown staining in the positive control. Histological and immunohistochemical samples were imaged with a slide scanner (Scanscope, Leica biosystems) and analyzed with the Aperio software (Leica biosystems).

5.9. Histological and immunohistochemical analyzes

Engineered tissue constructs were fixed in 4% paraformaldehyde, dehydrated in a graded series of ethanol’s, embedded in paraffin wax and sectioned at 5 μm. The sections were stained with hematoxylin and eosin to study cell morphology, alcin blue to reveal the presence of sGAG and picrosirius red to visualize the collagen content.

Collagen types I and II were also evaluated using a standard immunohistochemical technique. Rehydrated sections were treated with pronase (32 PUK/ml, Sigma-Aldrich) at 37 °C for 5 min, then incubated in blocking buffer containing 1% (w/v) BSA and 10% (v/v) goat serum (all from Sigma-Aldrich) in 1X PBS for 1 h at room temperature (RT) to block non-specific sites. Tissue sections were then incubated with type I collagen (Abcam, ref. 90,395, mouse monoclonal IgG, 1:400) or type II collagen (Santa-Cruz, ref. sc-52658, mouse monoclonal IgG, 1:200) primary antibody diluted in blocking buffer overnight at 4 °C in a humidified chamber. Samples were then incubated with 3% (v/v) hydrogen peroxide solution (Sigma-Aldrich) for 20 min to block endoperoxidase activity, then with secondary antibody (Sigma-Aldrich, ref. B7151, anti-Mouse IgG) diluted in blocking solution (1:200 for detection of type I collagen and 1:300 for detection of type II collagen) for 1 h at RT. Following a 45 min incubation period with ABC reagent (ABC Elite kit Vectastain PK-400, Vector Labs), the DAB substrate (SK-4100, Vector Labs) was added to the tissue section and the presence of the protein of interest was revealed by the apparition of brown staining in the positive control. Histological and immunohistochemical samples were imaged with a slide scanner (Scanscope, Leica biosystems) and analyzed with the Aperio software (Leica biosystems).

5.10. Polarized light microscopy

Rehydrated tissue sections were incubated at 37 °C for 18 h with 1000 U/ml bovine testicular hyaluronidase (Sigma-Aldrich, ref. H3506) prepared in 0.1 M phosphate buffer pH 6.9 to remove proteoglycans so birefringence was only caused by collagen fibrils [72,73]. Sections were then stained with 0.1% (w/v) picrosirius red, mounted with DPX (all from Sigma-Aldrich) and imaged with an Olympus BX41 polarizing light microscope equipped with a MicroPublisher 6™ CCD camera and an Olympus U-CMAD3 adaptor. Average orientation, dispersion and coherency of collagen fibrils in the engineered tissue and articular cartilage (12 weeks old pig, Danish Duroc, control sample) were assessed using orientationJ and directionality plugins in ImageJ [74].

5.11. Scanning helium ion microscopy

Engineered tissues were imaged with a scanning helium ion microscope (Zeiss ORION Nanofab) for high-resolution visualization of the collagen network. Before imaging, serial enzymatic digestion was used to remove glycosaminoglycans to provide an unobstructed view of the collagen fibrils; this protocol is based on the method described by Vanden Berg-Foels et al. [75]. The SHIM was operating with an acceleration voltage of 30 kV, a beam current of 1.54–1.72 pA, and a dwell time of 2–5 μs. Images were acquired using a pixel resolution of 1024 × 1024 or 2048 × 2048. The brightness and contrast were optimized and images were analyzed with orientationJ plugin in ImageJ; no other post-processing procedures were performed.

5.12. Mechanical testing

Unconfined compression tests were carried out on samples produced in the shape of a cylinder using a 5 mm diameter biopsy punch. Samples were placed in a PBS bath at room temperature (~25 °C) and compressed using a twin column Zwick universal testing machine (Zwick, Roell) equipped with a 100 N load cell. A preload of 0.02 N was used for empty scaffolds, whereas 0.5 N preload was applied to the tissue-engineered cartilage. A combined stress-relaxation and dynamic compression protocol was applied in increasing steps of 10% to a maximum of 30% [76]. Peak strain was reached within 500 s following 45 min relaxation. Five compressive cycles at 1% strain and 1 Hz frequency were then superimposed. The compressive (or ramp) modulus was taken as the slope of the stress-strain curve between 10%–20% and 20%–30% strain. The equilibrium modulus was determined for the last 10 s of the equilibrium phase following unconfined compression testing to 20% and 30% strain. The dynamic modulus was calculated from the average force amplitude over the five compression cycles following the relaxation test [42].

Stress-relaxation tensile tests for both the tissue-engineered constructs and PCL scaffolds were conducted on a TA Instruments TestBench with a 20 N load cell. The tensile samples were cut with a length to width ratio of 4:1 in the gauge section (20 mm × 5 mm) and an additional 5 mm for either grip section. The tests were characterized by an initial preload of 0.02 N and a sequential ramp through three relaxation points at 3, 6, and 9% strain. These points were shown to be below the yield point via uniaxial tensile testing. For each phase, a constant ramp rate of 0.3 mm/min and an equilibrium time of 30 min were used. After the final relaxation point, the test was ramped beyond the expected yield point to 40% strain. Throughout the test, samples were kept hydrated via PBS drips. Both the ramp and equilibrium tensile modulus were calculated for each relaxation phase. The ramp modulus was calculated as the slope of the stress-strain curve for linear regions approaching each equilibrium point. The equilibrium modulus was assessed by averaging the last 10 force readings from the load cell of each equilibrium phase. Finally, the yield point was calculated via an offset from the initial ramp modulus at 0.2% strain.

5.13. Statistical analyzes

Statistical analyzes were performed using the software package GraphPad Prism (Version 7.00). Statistical tests used to assess the normal distribution of data or to compare groups are indicated in figure legends. When groups were compared, significance was accepted at a level of p < 0.05. Results are expressed as mean ± standard deviation. Graphical results were produced with GraphPad Prism.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2022.121405.

References


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