Biofabrication of spatially organised tissues by directing the growth of cellular spheroids within 3D printed polymeric microchambers

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Abstract:

Successful tissue engineering requires the generation of human scale implants that mimic the structure, composition and mechanical properties of native tissues. Here, we report a novel biofabrication strategy that enables the engineering of structurally organised tissues by guiding the growth of cellular spheroids within arrays of 3D printed polymeric microchambers. With the goal of engineering stratified articular cartilage, inkjet bioprinting was used to deposit defined numbers of mesenchymal stem cells (MSCs) and chondrocytes into pre-printed microchambers. These jetted cell suspensions rapidly underwent condensation within the hydrophobic microchambers, leading to the formation of organised arrays of cellular spheroids. The microchambers were also designed to provide boundary conditions to these spheroids, guiding their growth and eventual fusion, leading to the development of stratified cartilage tissue with a depth-dependant collagen fiber architecture that mimicked the structure of native articular cartilage. Furthermore, the composition and biomechanical properties of the bioprinted cartilage was also comparable to the native tissue. Using multi-tool biofabrication, we were also able to engineer anatomically accurate, human scale, osteochondral templates by printing this microchamber system on top of a hypertrophic cartilage region designed to support endochondral bone formation and then maintaining the entire construct in long-term bioreactor culture to enhance tissue development. This bioprinting strategy provides a versatile and scalable approach to engineer structurally organised cartilage tissues for joint resurfacing applications.

1. Introduction:

Osteoarthritis is a degenerative joint disease affecting millions of people worldwide that is characterised by progressive loss of articular cartilage in the synovial joints ¹. Currently, surgical replacement of the diseased joint with a synthetic prosthesis is the only available treatment option for restoring some level of joint function in patients with end-stage osteoarthritis. While the procedure is well established, failures and complications are not uncommon ². As a result, there has been increased interest in using cartilage tissue engineering techniques to develop implants that can regenerate, rather than replace, a diseased joint. While significant progress has been made in the field, the majority of approaches have focused on the treatment of focal defects, and are not suitable for treating the diffuse damage present within an osteoarthritic joint. Biological resurfacing of such joints will likely require the engineering of tissues that recapitulate the stratified composition and architecture of adult articular cartilage ³. In particular, novel strategies are required to engineer cartilaginous tissues with biomimetic collagen networks, specifically fibers orientated parallel to the surface in the superficial zone and perpendicular to the surface in deeper zones ^{4,5}, as this architecture is integral to the biomechanical properties of the tissue ^{4,6}.

Engineering tissues that recapitulate the complex zonal organization of adult articular cartilage remains a significant challenge ⁷. Cartilage tissue engineering strategies often rely on homogeneously distributing chondrocytes or cartilage-progenitor cells throughout a biomaterial scaffold, and then stimulating these constructs with homogeneous biochemical and/or mechanical cues. As a result, engineered cartilage tissues are often homogenous in composition, lacking the complexity of their native counterparts. Different strategies to address these issues include the seeding of chondrocytes from the different zones of articular cartilage into layered hydrogels ⁸, the use of hydrogels with gradients in stiffness ⁹ or composition ^{10,11}, or modulating the environment through the depth of cell-laden hydrogels ¹². In a multifactorial approach, it was demonstrated how combinations of growth factors, substrate stiffness's and nanofiber alignments could be used to direct the zone specific chondrogenic differentiation of MSCs ¹³. In another approach, directed cell growth in multi-zonal scaffolds that mimicked the benninghoff architecture present in articular cartilage was shown to support the growth of tissues that recapitulate features of the native analogue ¹⁴. More recently, there has been increased interest in the use of 3D bioprinting in cartilage tissue engineering as the additive layer-by-layer nature of the technology

may be suitable for recreating the zonal organisation of the tissue. For example, introducing depth-dependent variations in cell density and cell type has enabled the engineering of cartilage tissues with regional distinctions in their composition ^{15,16}. However, fundamental challenges still exist, including the engineering of cartilaginous tissues that mimic the anisotropy and biomechanics of the native tissue, the development of constructs suitable for regenerating entire human-scale articular surfaces, and integrating bioprinted cartilage with an underlying bone region to permit integration within a diseased joint.

During early limb development stem cells aggregate and condensate before differentiating along a chondrogenic lineage. In fact, pellet culture, where mesenchymal stem cells (MSCs) are forced to aggregate using centrifugation, has been the standard culture system for initiation of stem cell chondrogenesis *in vitro* as it promotes cell-cell interactions that are analogous to those that occur during pre-cartilage condensation in early joint development ¹⁷. However, cartilage condensations developed in pellet culture systems are inherently small (<1 mm diameter) and lack the zonal organization of the native tissue, making them unsuitable for biological joint resurfacing. To overcome these limitations, a hybrid “bottom-up” approach could involve the printing of multiple cellular spheroids within a structural template capable of guiding their growth and fusion into scaled-up, structurally organised tissues ¹⁸. Here, we use cellular condensations as modular building blocks within 3D bioprinted implants, which enabled the engineering of scaled-up cartilaginous tissues mimicking the zonal organization of the native tissue. To achieve this, we developed a novel biofabrication strategy where defined numbers of MSCs are ink-jetted into 3D printed polycaprolactone (PCL) microchambers to promote initial cellular aggregation and eventual fusion. We demonstrate how such microchambers can be used to radially confine and direct the growth of the cellular aggregates, resulting in the development of scaled-up, stratified cartilage tissue. By integrating multiple-tool biofabrication with bioreactor technologies, we also demonstrate how this strategy can be extended to engineer osteochondral constructs mimicking the shape and complexity of human joints.

2. Results:

2.1 Inkjet printing of cells into 3D printed polymeric microchambers to induce spheroid formation

First, we developed a strategy for engineering arrays of cellular spheroids within printed polymeric microchamber templates. To do this, inkjet printing was used to deposit precise volumes of cell suspensions into confining hydrophobic polymeric microchambers to encourage cell condensation and spheroid formation (Fig 1 a). The microchamber system was formed by first printing a solid, non-porous, PCL base layer. Next, the microchamber walls (also non-porous) were generated by printing an orthogonal array of PCL microfibers (150 μm diameter) on top of the base layer (Fig 1 b). The microchamber height was kept constant throughout (0.6 mm), and two orthogonal fibre spacings were explored (0.8 mm and 1.2 mm), resulting in microchamber volumes of 0.25 mm³ and 0.66 mm³. Subsequently, the printed microchambers were loaded with cells by inkjet printing the cell suspension into each microchamber. As co-cultures of bone marrow derived mesenchymal stem cells (BMSCs) and chondrocytes (CC) have previously been shown to support the development of stable cartilage tissues resistant to hypertrophy and endochondral ossification *in vivo*¹⁹, we used inkjet printing technology to deposit a mixture of BMSCs and CCs (3:1 ratio) into the microchamber system. Two different microchamber cell seeding densities were explored by varying the inkjet valve opening time from 4 ms (0.635 $\pm$ 0.015 μl /droplet, ~20,000 cells per microwell) and 8 ms (1.24 $\pm$ 0.04 μl /droplet, ~40,000 cells per microwell) (Fig 1 c). Note, no hydrogel was used during inkjet printing, and cells were deposited while suspended in cell culture medium. Post-printing, the cell suspension was allowed to settle within the microchambers for 2 hours before adding excess media to the construct. As a control, a flat PCL base without microchambers was also inkjet seeded with cells (4 ms valve opening time). In addition, as a second control, a MSC:CC co-culture (150 μl and 300 μl ; 3:1 ratio CC:MSC; 30 million/ml) was manually pipetted onto the microchambers.

Post-printing, a structured array of cellular spheroids formed within the PCL template, indicating cellular condensation had occurred following deposition of the cell suspension into the microchambers. (Fig 1 d, e). Macroscopic and live/dead imaging indicated growth of the spheroids over time, and fusion between adjacent spheroids (over the PCL walls) was evident after 28 days

(Fig 1 d, e). For example, at day 0 cells are localised to the interior of the microchambers, and over 4 weeks of culture grow out of the microchambers leading to complete coverage of the top surface with viable cells (Fig 1 e). This indicated that the geometry of the microchambers was directing cells to migrate vertically out of the microchambers, which lead to eventual fusion of the cell aggregate over the microchamber walls. Cells did aggregate in the flat controls (no microchambers); however, the resulting tissues that formed by day 28 were random and unstructured (Supplementary figure 1). In the unprinted (manually seeded) microchamber controls, cells had not aggregated in the microchambers, but instead cells had preferentially coated the top surface of the PCL microfibers (Fig 1 d, e). Trapped air pockets within the microchambers indicated excess surface tension was preventing the cell suspension from entering the microchambers. As a result, the unprinted controls were not cultured past day 0. Taken together, these results demonstrate that structured arrays of cellular spheroids can be engineered by integrating inkjet printing technology and 3D printed microchamber templates.

Figure 1: Development of microchamber system and inkjet printing of cartilage tissue spheroids. (a) Schematic of inkjet bioprinting process where droplets of cell suspension were deposited in pre-printed PCL microchambers of defined geometry to induce cell condensation and spheroid formation. (b) Outline of different microchamber geometries (0.8 mm spacing, 1.2 mm spacing, and flat (no microchambers)), and schematic of inkjet seeding process. (c) Volume per droplet (μl) as a function of valve opening time (ms), and the resultant cell number based on 30 million/ml cell density. (d) Macroscopic and (e) live/dead images (z-stacks) of tissue formation in manually seeded and inkjet seeded samples at Day 0, 14, 28 of culture, scale bar 1mm.

After 4 weeks in chondrogenic culture conditions, we assessed the composition of the engineered cartilage that had formed within the PCL microchambers. After chemically dissolving the PCL using xylene, it was clear that the spheroids had started to fuse over the microchamber walls (Fig 2 a). The engineered tissue appeared thicker (more opaque) for the smaller

microchambers (Fig 2 a). In addition, the arrays of tissue pellets appeared more organized within the smaller microchambers (Fig 2 a). Biochemical quantification of changing DNA content confirmed cell proliferation had occurred in all groups over 4 weeks (Fig 2 b). The sGAG content of engineered tissues was within the range of native articular cartilage (>5 ww%), while histological staining for sGAG deposition confirmed robust cartilage formation within the microchambers (Fig 2 b). Finally, the compressive modulus (measured including PCL microchambers) of the engineered tissues were comparable to native cartilage values (Fig 2 b). The sGAG content of the engineered tissues was significantly higher when the microchamber spacing was increased from 0.8 mm to 1.2 mm for 20,000 cells/microchamber (Figure 2 b). However, it should be noted that the connective tissue which bound the cellular aggregates together appeared thicker for the smaller 0.8 mm spacing (Figure 2 a). In addition, the pellet organization was more structured and array-like (Figure 2 a). For this reason, the 0.8 mm spacing was brought forward to the next stage of the study. As the biochemical composition of the engineered tissues was comparable for both valve opening times, the lower seeding density of 20,000 cells per microchamber well (or 4 ms) was selected to reduce the total number of cells required. Together, these results demonstrate that arrays of bioprinted cellular spheroids can be used as modular building blocks to engineer larger scale cartilage tissues with native like biochemical and biomechanical properties.

Figure 2: Biochemical and biomechanical properties of the engineered cartilage tissue following 4 weeks of *in vitro* culture as a function of valve opening time and microchamber size. (a) Macroscopic images of aggregated tissue after dissolving the PCL framework, and corresponding histological for sGAG and (Side view, 4X scale bars 400 μ m). (b) DNA content (ng/construct), sGAG %ww, collagen %ww, and Young's modulus (measured including PCL microchambers) for each group after 28 days of culture. Unless directed connected with a line, * denotes significance compared to corresponding day 0 value, significance defined as ($p < 0.05$, ANOVA).

2.2 Multi-tool bioprinting of human-scale osteochondral templates

We next explored whether the cartilage microchamber system could be integrated with an underlying endochondral bone region to engineer scaled-up, multi-phase constructs suitable for

joint resurfacing. To do this, a three-step, multi-tool bioprinting approach was developed. This modular approach made it possible to 3D print a microchamber system capable of supporting cellular condensation on top of a PCL reinforced MSC laden hydrogel, which we have previously shown can support endochondral bone formation *in vivo*²⁰. The outer geometry of the joint resurfacing implant was designed using a μ CT scan of a human scale tibia (Fig 3 a). The first step of the biofabrication process involved printing a reinforcing PCL framework, which included a larger microchamber system to support articular cartilage development, and a porous PCL framework (fibre spacing 1.5 mm; fibre diameter 250 μ m) to mechanically support the underlying endochondral bone region (Fig 3 b). In the second step of the biofabrication process, the endochondral bone region was functionalised using a novel z-direction bioprinting approach, where alternating pores of the PCL framework were loaded with either a GelMA bioink containing MSCs (20 million/ml), or a sacrificial pluronic bioink which could be washed out post-printing (Fig 3 c & Video 1). Z-direction bioprinting involved depositing the required bioink by moving the needle in the z-direction through channels with the PCL framework. Following UV light mediated radical cross-linking of the GelMA component, the fugitive pluronic ink could be sacrificed in cold (4°C) culture media. This resulted in the formation of a network of bioink free microchannels which we have previously shown can support nutrient diffusion in larger scale tissues during bioreactor culture²¹. Finally, in the last step of the biofabrication process, the articular cartilage region was developed by inkjet printing a MSC:chondrocyte co-culture into the expanded microchamber system as described above (Fig 3 d & Video 1). This resulted in an MSC population in the endochondral bone region, and a MSC:CC co-culture cell population in the chondral region. It was also possible to integrate two PCL stems into the bone region which would help facilitate fixation in a joint (Figure 3 e). Thus, by integrating multiple biofabrication technologies, it is possible to regionally deposit different cell populations into pre-printed polymeric frameworks.

a Isolation of tibial plateau insert

b Fused Deposition Modelling PCL Framework

c Microextrusion bioprinting of Bone Region (Z-extrusion into pre-printed PCL scaffold)

d Inkjet bioprinting cartilage region

e

Figure 3: Multi-tool bioprinting of osteochondral implants. (a) Printing of integrated osteochondral PCL framework, scale 4 mm. (c) Step 1) Fused deposition modelling (FDM) printing of scaled-up PCL microchamber system. Step 2) FDM printing of PCL reinforcing framework for bone region. Step 3-5) Flexibility of printed PCL microchambers, and subsequent fusing of cartilage and bone regions to form PCL reinforced osteochondral template. (c) Microextrusion bioprinting of sacrificial pluronic component and GelMA/MSc bioink into PCL framework in bone region, live/dead analysis of MSc laden GelMA bioink including microchannels after washing out pluronics, scales 0.5 mm and 2 mm respectively. (d) Inkjet printing of MSc:CC suspension into microchamber system. (e) Design of unicompartmental joint prosthesis with fixation stems for use following tibial osteotomy. Macroscopic image of tibial shaped biological joint prosthesis after 28 days of *in vitro* culture.

2.3 Bioreactor culture enhances the *in vitro* development of scaled-up bioprinted tissues

The bioprinted constructs were then transferred to chondrogenic media and cultured in either static or dynamic conditions for 10 weeks using a custom developed bioreactor (Fig 4 a) ²¹. After 10 weeks of bioreactor culture, the articular cartilage and endochondral bone regions of the construct were separated, and we first assessed whether the MSc population in the bone region of the bioprinted prosthesis had independently become hypertrophic and proceeded along an endochondral pathway. Robust sGAG deposition was observed at the edge of the constructs for both dynamic and static culture conditions (Fig 4 b). As expected, dynamic culture enhanced sGAG production in core regions of the constructs (Fig 4 b). These histological observations were confirmed by biochemical analysis which revealed a significantly higher sGAG content in the bone region with dynamic culture (Fig 4 e). Type X collagen deposition, a marker of chondrocyte hypertrophy, was observed in this region of the bioprinted construct for both culture conditions (Fig 4 c-d). Compared to the overlying articular cartilage layer, significantly higher levels of calcium were also present in the endochondral bone region for both culture conditions (Fig 4 f). This indicated that the MScs in the bone region of these scaled-up bioprinted constructs were proceeding along an endochondral pathway, demonstrating the potential of integrating bioprinting and bioreactor culture for the engineering of scaled-up osteochondral constructs.

Figure 4: Outline of culture conditions, along with biochemical properties of the endochondral bone region following 10 weeks of *in vitro* culture. (a) Outline of static and dynamic culture conditions & macroscopic image of cultured implants (dynamic and static) after 10 weeks of culture, scale bars 5 mm. (b) Alcian blue staining for sGAG, 2X stitches, scale bars 1 mm. *Note, staining was performed at the mid-section of the constructs, approximately 4-6 mm from the bottom surface.* (c-d) Collagen II & X staining of the bone region for dynamic (c) and static (d) culture conditions; images 10X, scale bars 500μm. (e) sGAG content (%ww) and (f) Calcium content (%ww) (n=3). *denotes significance compared to

static culture for same region, # denotes significance to cartilage region for corresponding culture condition ($p < 0.05$, ANOVA).

2.4 Engineering of stratified articular cartilage on the surface of biological implants

We next assessed tissue formation within the articular cartilage region of the same bioprinted constructs after 10 weeks in either static or dynamic culture. Macroscopic imaging, along with live/dead staining, revealed extensive spheroid fusion had occurred in the articular cartilage region, and that a continuous layer of tissue covered the entire surface of the printed construct (Figure 5 a). Depth reconstruction renderings of the live/dead images demonstrated that the engineered tissues possessed a tibial-like curvature (Fig 5 a).

Figure 5: Tissue formation and spheroid fusion cartilage region following 10 weeks of *in vitro* culture. (a) Macroscopic images (top view) of pellet and tissue formation in the microchambers (scale-bar 5mm & 2mm), live dead staining of cells in the microchambers system (5mm), and depth reconstructions of the live dead images (scale-bar 5mm & 3mm), for dynamic and static culture conditions.

Macroscopic cross-sections of the cartilage layer revealed that an extensive layer of tissue had formed, and that over the duration of the culture period the spheroids had grown out of the microchambers leading to fusion across the microchamber walls (Fig 6 a). The tissue thickness approached 1 mm in both static and bioreactor culture (Fig 6 g). Histological staining for sGAG,

collagen II and lubricin confirmed that the microchambers were filled with hyaline cartilage-like tissue, and that the condensations had fused over the microchamber walls (Fig 6 b, c, f). Weak staining for collagen types I and X deposition further suggested the development of a phenotypically stable cartilage within the microchambers for both culture conditions (Fig 6 d, e). Biochemical analysis demonstrated that the engineered tissues possessed a native like sGAG content ($>8\%$ ww) (Fig 6 h), with bioreactor culture again enhancing overall levels of matrix deposition. Collagen content ($>4\%$ ww) approached but did not reach native values (Fig 4 i). Finally, the mechanical properties of the engineering tissues, assessed using unconfined compression testing, demonstrated that the equilibrium modulus was approximately 3000 kPa for both culture conditions (Fig 6 j), with the PCL microchambers likely contributing to construct mechanics. Together, these results demonstrate that the microchamber system can be expanded to direct the growth and fusion of hundreds of spheroids, thereby enabling the development of anatomically-shaped, human scale, cartilage tissues.

Figure 6: Biochemical and biomechanical properties of the engineered cartilage region following 10 weeks of *in vitro* culture. (a) Macroscopic cross-sections of the cartilage layers after 10 weeks of culture, scale bar 500μm. (b) Alcian blue staining for sGAG, 2X and 4X images, scale bars 500μm and 200μm respectively. (c) Collagen II staining, scale bar 200μm. (d) Collagen X staining, scale bar 200μm. (e) Collagen I staining, scale bar 200μm. (f) Lubricin staining, scale bar 200μm. (g) Histomorphometric analysis of cartilage layer thickness, (n=3 independent samples per group, multiple regions per sample analyzed). (h) sGAG content (%ww) and (i) Collagen content (%ww) in the engineered cartilage tissues (n=3). (j) 25% strain equilibrium modulus (n=3, per group). * denotes significance compared to static culture ($p < 0.05$, T-test).

Next, we assessed whether the boundary conditions provided by the microchambers had directed the growth of the cellular condensations and hence induced appropriate collagen network anisotropy within the scaled-up engineered tissue. To do this, polarized light microscopy was used

to assess the collagen fiber orientations in the engineered cartilage tissues. For both culture conditions, strong birefringence was observed at the superficial surface of the tissue, and along the microchamber walls (Fig 7 a). Colour mapping demonstrated that in the superficial zone, the collagen fibers were orientated parallel to the top surface, whereas in deeper zones the fibers were orientated more perpendicularly to the surface (Fig 7 a, b). The average fiber orientation was then assessed in the superficial, middle, and deep zones of the tissue. In the superficial zone the average fiber orientation varied around an average of $7.2 \pm 5.97^\circ$; in the middle zone, $53.2 \pm 16.2^\circ$; and in the deep zone $42.3 \pm 14.4^\circ$ (Fig 7 c). The coherency, which is used to assess the variance in the fiber distributions within each of the averaged regions, was higher in the superficial zone (Fig 7 d). These results demonstrate that by orientating the growth of the spheroids within the microchamber system it is possible to engineer scaled-up, articular cartilage tissues with native-like collagen fiber orientations. As already demonstrated (Fig 4), this occurred while MSCs in the underlying bone region were proceeding along an endochondral pathway, demonstrating that it is possible to spatially modulate MSC hypertrophy in order to engineer scaled-up osteochondral constructs suitable for joint resurfacing applications.

Figure 7: Collagen fiber orientations in the engineered cartilage region following 10 weeks of *in vitro* culture. (a) Polarized light and (b) colour map imaging of collagen fiber distributions in cartilage region. (c) Quantification of collagen fiber orientations and fiber coherency (representation of the variance in the fiber distributions within each of the averaged regions, for example a higher coherency value indicates a lower standard deviation in the measured average), in the deep, middle, and superficial zones (n=3, multiple regions analyzed per sample). # denotes significance compared to middle and deep zone for corresponding culture condition ($p < 0.05$, ANOVA). *Note, the superficial, middle, & deep zones were segregated by dividing the maximum tissue height into three and drawing a horizontal line across each images from these points.*

3. Discussion

A number of studies have demonstrated that bioprinting and/or biofabrication technology can be used to engineer mechanically reinforced hydrogels and cell-laden implants^{16,20,22–25}, however the structural complexity of native tissues has not been recreated. Here, we describe a novel biofabrication approach for engineering mechanically functional cartilage tissues with a native-like collagen network. Using multi-tool biofabrication, we developed a microchamber system that could support the formation of organized arrays of mesenchymal condensations. Over time, these modular condensations acted like building blocks which fused to form larger cartilage tissues. By orientating the growth of these cellular aggregates in the microchamber system, it was also possible to produce cartilage tissues with a collagen network organization mimicking the native tissue; specifically a parallel orientation in the superficial zone and random-perpendicular organization in the middle and deep zones. Importantly, the sGAG content and biomechanical properties of the tissue were also in the range of native cartilage. It was also possible to integrate the inkjet printing process with microextrusion bioprinting, to engineer a scaled-up biological joint prosthesis containing a curved layer of stratified articular cartilage, and an underlying endochondral bone region composed of hypertrophic cartilage. Dynamic bioreactor culture was found to enhance matrix accumulation in both the cartilage and endochondral bone regions, enabling the production of clinically sized biological implants over 10 weeks of culture. Taken together, these results demonstrate how the integration of a number of different biofabrication and bioreactor strategies can make it possible to produce human-scale, stratified cartilage tissues for joint resurfacing applications.

The integration of inkjet printing and pre-printed PCL microchambers made it possible to engineer structured arrays of mesenchymal condensations. Inkjet printing overcame surface-tension limitations observed with manual seeding, and made it possible to rapidly engineer large arrays of cellular spheroids on curved, anatomical geometries. This would not have been possible using regular centrifugation or manual seeding. The microchamber system not only facilitated the formation of mesenchymal condensations, but also acted to orientate their growth, thereby enabling the engineering of stratified cartilage tissues with biomimetic collagen fiber orientations. The structured organization is likely due to the orientated growth enforced by the boundaries of the microchambers. It has previously been shown that radially confining the growth of self-

assembling cartilage tissues can enhance collagen fiber organization in the direction perpendicular to the articular surface ²⁶. The authors hypothesized the enhanced collagen fiber organization was due to internal stresses created by the confined growth of the tissue. It is also possible that cell migration, which was directed along the microchamber walls, influenced the organization of matrix components during tissue self-assembly. It is well established that cells can reorganize ECM components during collective migration ²⁷. In addition, it has recently been shown that 3D-printed PCL microfibers can guide the growth of collagenous tissues *in vivo* ²⁸. It is clear from live/dead staining over the course of the 4 week culture period that cells are initially localised in the interior of the microchambers, and subsequently migrate out of the microchambers and fuse with adjacent spheroids over the top of the microchamber walls (Fig 1 d, e). After longer term culture, this leads to significant ECM production over the microchamber walls (Fig 6 a-f). The parallel collagen fiber orientation present in the superficial zone is likely due the directed cell migration that occurred over the top of the microchamber walls during fusion of the cartilage tissues aggregates. It should also be noted that along with the directing collagen fiber orientation, the microchamber system facilitates integration of the cartilage and bone regions. For example, it is challenging to integrate self-assembled cartilage onto flat 2D surfaces (supplementary figure 1). Addressing the challenge of cartilage-bone region integration is particularly important *in vivo* where immediate resistance to shear loading is required. Future studies, will explore the benefits of the microchamber system in pre-clinical osteochondral defect models.

The strategy developed in this study to generate scaled-up osteochondral implants offers several advantages over previous tissue engineering and 3D bioprinting approaches to joint regeneration. As mentioned previously, a number of studies have explored the use of different bi-phasic and tri-phasic scaffolds for cartilage and osteochondral defect repair ^{14,29–32}. While these approaches are promising for treating focal defects, challenges will arise when attempting to scale-up such approaches up to treat whole articular surfaces. This had led to increased interest in bioprinting, as the technology makes it possible to engineer geometrically complex hydrogels reinforced with a stiff polymeric phase ^{22,24,33–35}. In addition, a number of studies have explored whether the additive layer-by-layer nature of bioprinting technology can be leveraged to engineer cartilage tissues with regional distinctions in their composition ^{15 16}. For example, bioprinting technology has been used to bioprint bi-layered chondral constructs, composed of an articular chondroprogenitor cell (ACPC) laden bioink in the superficial zone, and an MSC laden bioink in

the deep zone¹⁶. After 8 weeks of culture in chondrogenic conditions, each region possessed a distinct cellular and extracellular matrix composition. Here, we have built on these studies by incorporating a reinforcing PCL component into the tissue that not only improves mechanical properties, but also simultaneously directs the alignment of newly deposited extracellular matrix to produce a cartilaginous tissue with a native-like collagen fiber architecture. In addition, articular surfaces are not flat and possess distinct curvatures depending on location. Our approach enabled the formation of stratified cartilage tissues that possess the natural curvature of the tibial plateau, which is a key consideration for joint resurfacing applications.

The utility of biofabrication technology was further demonstrated by integrating inkjet and microextrusion bioprinting to control the placement of 3 different components (1: MSC-chondrocyte co-culture; 2: MSC laden bioink; 3: pluronics) within a pre-printed, reinforcing PCL framework (Fig 3 b-d). In the endochondral bone region of the implant, this made it possible to locally deposit pluronics or an MSC laden bioink within alternating channels of the reinforcing PCL framework. Such control would not have been possible without biofabrication technology. Tissue engineering a human scale biological joint prosthesis also requires satisfying the physiochemical demands of large volumes of cells. Nutrient and oxygen diffusion limitations that arise during 3D culture can lead to the development of spatially inhomogeneous cartilage tissues with core regions devoid of matrix³⁶⁻³⁸. These limitations become exacerbated when scaling-up engineered tissues. We found that it was possible to at least partially address these challenges and engineer tissues of scale by modifying the architecture of the bioprinted construct and introducing dynamic bioreactor culture. Specifically a novel dual z-bioprinting approach was developed where alternating pores of a pre-printed PCL scaffold were simultaneously filled with two inks (GelMA/MSC and pluronic). The fugitive pluronic ink was then washed away, leaving behind a network of microchannels in the endochondral bone region to support enhanced nutrient transfer³⁹⁻⁴¹. Dynamic bioreactor culture was shown to enhance sGAG production (4.24% versus 1.89%) in core areas of the bone region, whereas with static culture, large areas were completely devoid of matrix. Improvements in tissue formation were less pronounced in the cartilage region of the construct. This is likely due to the lower thickness of the cartilage region (700 μ m) compared to the bone region (5 mm at the mid-section), which attenuated nutrient diffusion limitations. However, it should still be noted that sGAG production was significantly improved with dynamic culture (12.3% versus 8%) (Fig 6 h), and there were trends towards higher tissue thickness,

collagen production, and mechanical properties (Fig 6 g, i, j). Overall, these results suggest that the application of dynamic bioreactor culture is beneficial when attempting to tissue engineer human-scale constructs for joint resurfacing applications.

Osteoarthritis is a disease that affects the articular cartilage as well as the underlying bone. Therefore, a biological prosthesis must be capable of regenerating both tissues within the joint. In this study, a developmentally inspired approach is used where MSC hypertrophy is spatially regulated in order to engineer stable articular cartilage overlaying transient hypertrophic cartilage. In the articular cartilage region weak staining for collagen X was present, whereas in the bone region, the MSCs had proceeded along an endochondral pathway as evidenced by strong collagen X staining and enhanced calcium deposition (Figure 4 & Figure 6). This indicates that the chondrocyte population in the cartilage region was locally suppressing hypertrophic differentiation of the MSC population. It has been demonstrated that paracrine factors such as parathyroid - hormone-related protein (PTHrP), released by articular chondrocytes, can suppress hypertrophy of MSCs in co-culture ⁴²⁻⁴⁵. There is also evidence to suggest that MSCs will drive the proliferation of chondrocytes, while MSCs in the same co-culture reduce in frequency, eventually resulting in a tissue predominately containing phenotypically stable chondrocytes ⁴⁶. The ability to spatially regulate chondrocyte hypertrophy using different populations of cells is particularly advantageous as the bioprinted constructs can then be cultured using a single culture media. This alleviates the need to develop complex, dual-chamber culture systems which can be challenging to integrate with dynamic bioreactors. While this developmentally inspired framework has been reported before ¹⁹, previous attempts have used soft hydrogels to engineer the bi-layered cartilage tissues, which are unsuitable for joint resurfacing applications. In this study, the adoption of multi-tool biofabrication made it possible to reinforce the immature cartilage tissues with a stiff PCL framework. This made it possible to decouple the biological and mechanical functionality of the implant. The mechanical properties in the articular cartilage region were within the range of native values, whereas we have previously shown that the mechanical properties of PCL reinforced bioinks are within the range of trabecular bone ⁴⁷. It is anticipated that this mechanical reinforcement would help the implant withstand immediate load bearing in a joint environment.

PCL was used as the reinforcing material throughout our bioprinted implant. Its favourable extrusion characteristics enable the 3D printing of anatomically shaped implants. In addition, it

has been demonstrated that 3D printed PCL scaffolds are sufficiently mechanically robust to withstand articulation following implantation into the humeral condyle in a model of whole joint regeneration in skeletally mature rabbits ⁴⁸. This suggests that implants reinforced with 3D printed PCL, such as that developed here, can be engineered to be sufficiently robust for use in large chondral and osteochondral defects. Future work is however required to better characterise the mechanical properties of the PCL frameworks and the engineered cartilaginous tissues within, and in particular to determine (1) how the properties of the engineered cartilage varies through the depth of the construct, and (2) how well it is integrated into the PCL microchambers. It should also be noted that the degradation rate of PCL microfibers is slow, and it typically takes 2-3 years before the polymer is fully degraded ^{49,50}. Ideally, the rate of PCL degradation would be tailored to match the rate of tissue maturation to enable long term restoration of the articular surface. Future work (both computational and experimental) will look to determine the optimum initial mechanical properties required to support joint stability. Once this has been identified, the degradation rate of the reinforcing PCL phase will also be tailored to match to neo-tissue formation. Methods are available for tailoring the degradation rate of polymers such as PCL ⁵¹, which are compatible with the framework we have presented in this study.

4. Conclusion:

In this series of experiments we have established a novel biofabrication framework that enabled the engineering of stratified cartilage tissues for joint resurfacing. To date, the majority of cartilage tissue engineering approaches have focused on developing scaffolds or hydrogels to treat focal defects, and are not suitable for treating an osteoarthritic joint. In addition, a major challenge in the field has been recapitulating the stratified zonal architecture present in adult tissue. Here, we have utilised 3D printing to address both of these challenges, by (1) printing scalable arrays of spheroids within a reinforcing polymeric framework, and (2) subsequently using the polymeric framework to orientate spheroid growth and fusion to engineer scaled-up tissues with native-like collagen anisotropies. In addition, we were also able to engineer a human scale tissue by integrating bioprinted microchannels and bioreactor technology into the framework. Overall, the approach has a number of advantages over traditional top-down biofabrication approaches, and we anticipate that such bioprinted implants could offer an alternative treatment option to synthetic joint replacement prosthesis.

5. Methods

5.1 Isolation and expansion of MSCs and chondrocytes

Bone marrow derived MSCs were isolated from the femoral shaft of 4 month old pigs (Danish Duroc, female) and expanded in high glucose dulbecco's modified eagle's medium (hgDMEM) GlutaMAX supplemented with 10% v/v FBS, 100 U/ml penicillin, 100 µg/ml, streptomycin, 2.5 µg/ml amphotericin B at 5% pO₂ (all Gibco, Biosciences, Dublin, Ireland). Following colony formation, MSCs were trypsinized, counted, seeded at density of 5000 cells cm² in 500 cm² triple flasks (Thermo Fisher Scientific), supplemented with hgDMEM, 10% v/v FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 5 ng/ml human fibroblastic growth factor-2 (FGF-2; Prospec-Tany TechnoGene Ltd., Israel) and expanded to passage 2 at 5% pO₂. Tripotentiality was confirmed prior to use as previously described ⁵². Articular cartilage was aseptically harvested from porcine femoral condyles (Danish Duroc, female, 4 months old) and the cartilage slices were rinsed thoroughly with Dulbecco's phosphate buffered saline (PBS) containing penicillin (200 U/mL)-streptomycin (100 µg/mL) and amphotericin B (2.5 µg/mL). Chondrocytes (CCs) were isolated from cartilage slices overnight by digestion with high-glucose Dulbecco's modified Eagle's medium GlutaMAX (4.5 mg/mL D-Glucose, 200 mM L-Glutamine; hgDMEM) containing collagenase type II (350 U/mL) for 14 h under constant rotation at 37 °C. The resulting cell suspension was passed through a 40 µm pore-size cell sieve and the filtrate centrifuged and rinsed with PBS twice. Cell number and viability were determined using a haemocytometer and 0.4 % trypan blue staining.

5.2 GelMA synthesis

Gelatin methacryloyl (GelMA) was synthesized by reaction of porcine type A gelatin (Sigma Aldrich) with methacrylic anhydride (Sigma Aldrich) at 50 °C for four hours, as previously described ⁵³. Methacrylic anhydride was added to a 10% solution of gelatin in PBS under constant stirring. To achieve a high degree of functionalization, 1 ml of methacrylic anhydride was added per gram of gelatin. This protocol has been shown to support a degree of metacrylation of approximately 75% ^{54,55}. The functionalized polymer was dialyzed against distilled water for 7

days at 40 °C to remove methacrylic acid and anhydride, freeze-dried, and stored at -20 °C until use.

5.3 *In vitro* culture conditions

The chondrogenic culture conditions applied in this study are defined as culture in a chondrogenic medium (CDM) consisting of hgDMEM GlutaMAX supplemented with 100 U /ml penicillin, 100 µg/ml streptomycin (both Gibco), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 4.7 µg/ml linoleic acid, 1.5 mg/ml bovine serum albumine, 1 X insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma–Aldrich), 2.5 µg ml⁻¹ amphotericin B and 10 ng/ml of human transforming growth factor-β3 (TGF-β3) (Prospec-Tany TechnoGene Ltd., Israel) at 3% pO₂ or 20% pO₂.

5.4 3D-bioprinting system (FDM, microextrusion and inkjet printing)

All constructs were bioprinted using the 3D Discovery multi-head bioprinting system purchased from Regen Hu, Switzerland. The 3D Discovery contains a fused deposition modelling (FDM) printhead for melt extrusion of polymers, a microextrusion printhead for contact dispensing of cell laden bioinks, and an inkjet printhead for contactless dispensing of cell laden bioinks (supplementary figure 2). PCL (Sigma, Mn 45,000) scaffolds were printed for the cartilage and bone regions using the parameters described in supplementary table 1. All bioprinting parameters are also provided in supplementary table 2. 3D printing g-code toolpaths were generated using BioCAD software when possible (RegenHU, Switzerland). [To facilitate z-extrusion in the pre-formed PCL scaffolds, custom g-codes were generated.](#)

To produce an array of microchambers, a solid, non-porous, base (thickness ~100µm) was first printed using PCL. Next, two different microchamber geometries were generated by printing an orthogonal array of PCL microfibers (150 µm diameter), with two filament spacings (0.8 mm and 1.2 mm), on top of the base (Figure 1 b). Two different cell seeding densities were explored by varying the inkjet valve opening time from 4 ms (0.635 ± 0.015 µl/droplet, ~20,000 cells) to 8 ms (1.24 ± 0.04 µl/droplet, ~40,000 cells) (Figure 1 c). The printhead cartridge was changed every 15 minutes to prevent excess cell settling. The inkjet printhead was aligned with the underlying microchambers by positioning the printhead tip directly above the centre of a single microchamber.

Next, the printhead was translated vertically in the z-axis by 40 mm. The printing path (alternating horizontal paths, 0.8mm or 1.2mm spacing) was generated using the dimensions of the outer perimeter of the construct, and a single droplet was deposited every 0.8 mm or 1.2 mm at a constant speed of 4 mm/s to fill the microchambers. Note, the distance between the tip of the inkjet printhead and the top surface of the microchambers was kept constant at 40 mm during printing

To produce larger scale osteochondral constructs a multi-tool biofabrication framework was established to bioprint an anatomically accurate, human scale, tibial shaped implant (Figure 3). First, a reinforcing PCL framework was printed, which included a larger microchamber system (microchamber spacing 0.8 mm) to support articular cartilage development, and a porous PCL framework (fibre spacing 1.5 mm; fibre diameter 250 μ m) to mechanically support the underlying endochondral bone region (Figure 3 b). The fiber architecture was designed as orthogonal throughout. Due to the relatively thin nature of the printed microchambers, it was possible to press mold the them onto the curved surface of the printed bone region, by heating the underside of the base for 15 seconds (80°), and pressing the two regions together to facilitate PCL fusion (Figure 3 b). The combined osteochondral implant was then aligned on the printing platform using a mold in the shape of the bone region (supplementary figure 3). Subsequently, the bone region was functionalised using a novel z-direction bioprinting approach, where alternating channels of the scaffold were loaded with either a GelMA bioink containing MSCs (20 million/ml), or a sacrificial pluronic bioink which could be washed out post-printing (Figure 3 c). The GelMA cell suspension was prepared by mixing 20 wt % GelMA (with 0.1 wt % irgacure, dissolved in hgDMEM) in a 1:1 ratio with MSCs (40 million/ml in expansion media). After the printing, the GelMA component was cross-linked using UV light for 25 mins (365nm, 5 mW/cm²). After crosslinking, the fugitive pluronic could be washed out with cold (4°C) culture media. This resulted in the formation of a PCL/GelMA composite, containing a network of bioink free microchannels to support nutrient transport during *in vitro* culture. Next, the construct was inverted and inkjet printing was used to seed the microchamber system (0.8 mm spacing, 30 million/ml density) in the cartilage region as before (Figure 3 d). Note, the construct was carefully aligned on the printing platform to ensure all of the microchambers were equally filled throughout the construct, and the inkjet printhead was programmed to translate at a constant speed of 4 mm/s over the the microchambers and a single droplet was deposited every 0.8 mm. The printing path (alternating horizontal paths, 0.8mm spacing) was generated using the dimensions of the outer perimeter of the construct. A two hour

settling time was used to allow cell aggregation in the microhambers. Note, during the two hour settling time the bone region was submerged in cell culture media. The printed constructs were then transferred to chondrogenic media and cultured in either static or dynamic conditions for 10 weeks using a custom developed bioreactor (Figure 4 a). The external oxygen tension was kept low (3% pO₂) for the first 4 weeks to promote chondrogenesis in both regions, and then switched to 20% pO₂ for the final 6 weeks to promote hypertrophic differentiation of the MSC population in the endochondral bone region.

5.5 Biochemical analysis

The biochemical content of constructs was analysed after 10 weeks of culture. The bone and cartilage regions were separated and an 8 mm biopsy punch was used to isolate a sample from each region for biochemical analysis. Prior to biochemical analysis, constructs were washed in PBS, weighed, and frozen for subsequent assessment. Each construct was digested with papain (125 µg ml⁻¹) in 0.1M sodium acetate, 5mM L-cysteine–HCL, 0.05 methylenediaminetetraacetic acid (EDTA), pH 6.0 (all from Sigma–Aldrich) at 60 °C and 10 rpm for 18 h. DNA content was quantified using the Hoechst Bisbenzimidazole 33258 dye assay, with a calf thymus DNA standard. The amount of sulphated glycosaminoglycan (sGAG) was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. To exclude any background absorbance from the GelMA the PH of the DMMB was adjusted to 1.35. Total collagen content was determined by measuring the hydroxyproline content using the dimethylaminobenzaldehyde and chloramine T assay, and a hydroxyproline to collagen ratio of 1:7.69. Another construct was digested in 1 M HCL and 60° C and 10 rpm for 18 h. The calcium content was determined using a Sentinel Calcium kit (Alpha Laboratories Ltd, UK). Note, the weight of PCL was excluded from all tissue wet weight (ww%) normalisations.

5.6 Histological and immunohistochemical analysis

Constructs were fixed in 4% paraformaldehyde, dehydrated in a graded series of ethanol's, embedded in paraffin wax, sectioned at 10 µm, and affixed to microscope slides. The sections were stained with alcian blue to assess sGAG content and picrosirius red to assess collagen content. Picrosirius stained sections were visualized under polarization to assess the collagen fibre

orientations in the engineered tissues. The collagen fibre orientations were assessed using orientation-J, an image-j plugin for directionality analysis in images ⁵⁶.

Collagen types I, II and X were evaluated using a standard immunohistochemical technique as described previously ^{47,57}. Lubricin staining was performed using a similar approach. Sections were treated with pronase (32 PUK/ml, Sigma-Aldrich) in a humidified environment at 37°C to enhance permeability of the extracellular matrix. Sections were then incubated with goat serum to block non-specific sites for 2 hours, before the primary antibody was applied to the sections. Anti-PRG4 (1:50, Novus Biologicals H00010216-M01, mouse monoclonal IgG2a), primary antibody was applied overnight at 4°. Thereafter the sections were treated with peroxidase followed by the secondary antibody (Anti-Mouse IgG biotin conjugate, 1:200) was added for 1 hour followed by incubation with ABC reagent (Vectastain PK-400, Vector Labs, Peterborough, UK) for 45 min. Finally sections were developed with DAB peroxidase (Vector Labs) until brown staining was observed in the positive control. Positive and negative controls were included in the immunohistochemistry staining protocol for each batch. Note, if brownish staining was observed in the negative controls for this time period the staining was repeated until corrected.

5.7 Live/dead confocal microscopy

Cell viability was assessed after using a LIVE/DEAD™ viability/cytotoxicity assay kit (Invitrogen, Bio-science, Ireland). Briefly, constructs were washed in PBS followed by incubation in PBS containing 2 µM calcein AM (green fluorescence of membrane for live cells) and 4 µM ethidium homodimer-1 (red fluorescence of DNA for dead cells; both from Cambridge Bioscience, UK). Samples were again washed in PBS, imaged with a Leica SP8 scanning confocal at 515 and 615 nm channels, and analysed using Leica Application Suite X (LAS X) software. All images presented are 3D z-stack reconstructions analysing cell viability through the depth of the tissue (view is from the top, 800-1000µm deep). The depth reconstruction images were produced by rendering the 3D live dead montage with Imaris software (Bitplane).

5.8 Mechanical characterization

Samples were tested in unconfined compression as previously described ²⁰. For all mechanical testing a biopsy punch was used to create 5 mm Ø cylindrical construct. The young's

modulus was assessed using stress tests where the samples were compressed with a ramp displacement of 1 mm/s until 10% strain. The compressive modulus was taken as the slope of the stress strain curve between 0%–10% strain. The equilibrium compressive modulus was determined from the equilibrium force following unconfined compression testing to 25% strain.

5.9 Bioreactor Culture

The smaller scale constructs developed in figures 1 were cultured in 24 well plates, with 2mls of CDM media added per construct (3% pO₂). All large scale osteochondral constructs (figure 3 onwards), were cultured in a custom made bioreactor system which has been described previously ²¹. The constructs were continuously translated at a speed of 0.05 mm/s and an amplitude of 6 mm for the entire culture period. As a static control, printed constructs were placed in an identical housing system except the actuator was not translated. Constructs were cultured in static and dynamic conditions for 4 weeks at an external oxygen tension of 3% pO₂, followed by 6 weeks at an oxygen tension of 10% pO₂ (both controlled in the incubator). Media (400 mls per cell culture bath) was changed twice weekly.

5.10 Statistical analysis

Statistical analyses were performed using the software package GraphPad Prism (Version 6.02). Groups were analysed by a general linear model for analysis of variance with groups of factors. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $p \leq 0.05$. Numerical and graphical results are presented as mean $\pm$ standard deviation, with graphical results produced using GraphPad. Student t-tests were used when comparing two groups.

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