Reinforcing interpenetrating network hydrogels with 3D printed polymer networks to engineer cartilage mimetic composites

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Abstract

Engineering constructs that mimic the complex structure, composition and biomechanics of the articular cartilage represents a promising route to joint regeneration. Such tissue engineering strategies require the development of biomaterials that mimic the mechanical properties of articular cartilage whilst simultaneously providing an environment supportive of chondrogenesis. Here three-dimensional (3D) bioprinting is used to develop polycaprolactone (PCL) fibre networks to mechanically reinforce interpenetrating network (IPN) hydrogels consisting of alginate and gelatin methacryloyl (GelMA). Inspired by the significant tension-compression nonlinearity of the collagen network in articular cartilage, we printed reinforcing PCL networks with different ratios of tensile to compressive modulus. Synergistic increases in compressive modulus were observed when IPN hydrogels were reinforced with PCL networks that were relatively soft in compression and stiff in tension. The resulting composites possessed equilibrium and dynamic mechanical properties that matched or approached that of native articular cartilage. Finite Element (FE) modelling revealed that the reinforcement of IPN hydrogels with specific PCL networks limited radial expansion and increased the hydrostatic pressure generated within the IPN upon the application of compressive loading. Next, multiple-tool biofabrication techniques were used to 3D bioprint PCL reinforced IPN hydrogels laden with a co-culture of bone marrow-derived stromal cells (BMSCs) and chondrocytes (CCs). The bioprinted biomimetic composites were found to support robust chondrogenesis, with encapsulated cells producing hyaline-like cartilage that stained strongly for sGAG and type II collagen deposition, and negatively for type X collagen and calcium deposition. Taken together, these results demonstrate how 3D bioprinting can be used to engineer constructs that are both pro-chondrogenic and biomimetic of the mechanical properties of articular cartilage.

Keywords: articular cartilage, tissue engineering, interpenetrating network hydrogels, polycaprolactone, 3D bioprinting, finite element modelling, biomimetic scaffolds

1. Introduction

Articular cartilage is a thin layer of connective tissue that lines the ends of bones within synovial joints. Under normal physiological conditions, cartilage provides a smooth, lubricated surface for articulation that facilitates load support

and distribution across the joint. Biomechanically it functions as a multiphasic, fibre-reinforced material that has anisotropic, nonlinear and viscoelastic properties [1-3]. The complex load bearing properties of articular cartilage strongly depend on the anisotropic nature of the tissue, consisting primarily of collagen and proteoglycans, and the associated tension-compression nonlinearity [1, 2, 4, 5]. The negatively charged

proteoglycans within articular cartilage generate an osmotic swelling pressure, which is balanced by a pre-stress that is developed in the collagen network [3, 6]. The compressive properties of cartilage typically increase as the proteoglycans content increases, and the loss of proteoglycans has been shown to make the tissue more prone to microdamage during mechanical loading [1, 7-10]. The architecture and organization of the collagen fibres determine the intrinsic tensile stiffness and strength of cartilage [10-12]. The tensile modulus of articular cartilage is significantly higher than the compressive modulus [13, 14]. When cartilage is tested in unconfined compression, this tension-compression nonlinearity enhances fluid pressurization and elevates the dynamic modulus of the tissue [15, 16], as the collagen network restricts lateral expansion of the tissue during compressive loading. The relative importance of the different matrix components on the overall mechanical properties of articular cartilage has been studied by depleting cartilage samples of collagen and/or proteoglycans [2, 9, 10, 17]. For example, the compressive modulus of the proteoglycan-depleted cartilage matrix may be as low as 3 kPa, representing less than 2% of the normal tissue modulus [18]. Therefore, the collagen in articular cartilage can be considered as a fibrillar matrix that primarily sustains tension. Biomaterial strategies that aim to mimic articular cartilage should consider such complexities in their design.

As articular cartilage has poor regenerative capacity, damage to this tissue impairs its mechanical function and can lead to an abnormal loading within the joint. If left untreated, the damage can progress to osteoarthritis of the joint, causing significant pain and disability. This motivates the need for new regenerative approaches capable of restoring the normal biomechanics of the joint.

A number of tissue engineered strategies to engineer cartilage replacements have been developed [19-27]. A typical approach is to design biomaterial environments capable of supporting chondrogenesis; this commonly involves combining cells and/or bioactive molecules with hydrogels as a scaffolding material due to their high water content and attractive transport properties [28-31]. Other than presenting pro-chondrogenic biological cues to the encapsulated cells, such biomaterial scaffolds must also provide a suitable mechanical environment capable of instructing cell fate as well as withstanding the challenging physiological loading conditions experienced *in vivo* upon implantation into the body [27]. Commonly used single network hydrogels (e.g. alginate, GelMA, agarose, polyethylene glycol, etc.) have been extensively studied and have been shown to provide an environment that is conducive to chondrogenesis. However, they are typically mechanically weak and unable to withstand the challenging environment of the joint. Recently, more complex hydrogel systems such as IPN hydrogels, characterized by the combination of multiple polymer

networks have been investigated. Within such IPN hydrogels individual polymer networks are physically entangled with each other and crosslinked only with themselves using specific chemistries [32-35]. In general, such IPN hydrogels have displayed synergistic increases in mechanical properties, including increased toughness and elasticity when compared to the single hydrogel components. However, they do not mimic many of the features of load bearing tissues like articular cartilage, such as anisotropy and tension-compression nonlinearity. Furthermore, engineering IPN hydrogels with bulk mechanical properties approaching that of articular cartilage typically requires very high hydrogel polymer concentrations, resulting in biomaterial environments that are not compatible with robust extracellular matrix (ECM) deposition by encapsulated cells [36-38].

To address these concerns, hydrogels can be reinforced with networks of fibres to produce composites with improved mechanical properties [2, 31, 39-41]. However, these techniques typically involve the use of complex biofabrication strategies (e.g. weaving, melt electrowriting) that are challenging to scale and personalize to specific patient anatomies. In contrast, Fused Deposition Modelling (FDM) can be used to 3D print fibre networks that are relatively simple to scale, with internal and external architectures that are easily defined based on medical imaging [42, 43]. This is achieved using PCL, a synthetic thermoplastic polymer which has been extensively used in the field of 3D bioprinting [44]. Advantages include ease of shaping and manufacture due to its low melting temperature ($\sim 60^{\circ}\text{C}$), biocompatibility, elasticity and mechanical properties [45]. The mechanical properties of PCL scaffolds can also be tuned by modifying the molecular weight and the scaffold architecture [46]. Other synthetic polymers such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) are also biocompatible with FDM, with PLGA also allowing for more tailorable degradation rates [47-49]. A concern with the use of PLGA is the release of acidic products as the polymer degrades, which can induce inflammation [50, 51]. Another major disadvantage is the higher melting temperature of PLA and PLGA compared to PCL, which is not ideal when co-printing with live cells.

In this study, alginate – GelMA IPNs were reinforced with PCL networks printed using FDM to develop biomechanically competent composite constructs for cartilage tissue engineering. [30, 52]. Alginate is a widely used biomaterial in tissue engineering because of its excellent biocompatibility, relatively high control over stiffness and capability to form highly porous structures which facilitate the diffusion of large molecules in and out of the hydrogel [53-55]. However, alginate is often limited under mechanical loading due to its brittleness and low elasticity [56-58]. GelMA is a gelatin-based material that has high biocompatibility, degrades in response to matrix metalloproteinases (MMP), possesses

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2
3 RGD sequences that favour cell adhesion and is highly elastic
4 [59, 60]. GelMA also possesses relatively poor mechanical
5 strength and toughness [59, 61, 62], however IPNs of alginate
6 and GelMA have previously been shown to have superior
7 mechanical properties to their individual hydrogel
8 components and to be supportive of cell growth and
9 differentiation [59]. Here, alginate, GelMA and alginate –
10 GelMA IPN hydrogel scaffolds were first mechanically
11 characterized to investigate the benefit of combining the two
12 single hydrogel components for engineering cartilage-mimetic
13 hydrogels. These hydrogel bioinks were then reinforced with
14 3D printed PCL networks with architectures and mechanical
15 properties inspired by the collagen network of articular
16 cartilage. The reinforcement mechanism resulting from the
17 combination of PCL networks with IPN hydrogels was
18 examined using Finite Element Analysis (FEA). Finally, the
19 ability of 3D bioprinted cell-laden PCL reinforced IPN
20 constructs to support chondrogenesis was assessed *in vitro*
21 over 6 weeks of culture.

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26 **2. Materials and methods**

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28 **2.1 Materials preparation**

29
30 Alginate was prepared by dissolving Alginate (PRONOVA
31 UP LVG) in DMEM to make up a final concentration of 3.5%
32 w/v. Cylindrical constructs were obtained by pouring the
33 alginate solution into 2 mm high-6 mm diameter agarose-
34 CaCl₂ moulds (DMEM, 3% w/v agarose, 45 mM CaCl₂) and
35 allowed to crosslink for 20 min at room temperature. GelMA
36 synthesis was previously described [63]. Briefly, GelMA was
37 synthesized by reaction of porcine type A gelatin (Sigma-
38 Aldrich, average molecular weight 40-50 kDa) with
39 methacrylic anhydride (Sigma-Aldrich) at 50 °C for 4 h. To
40 achieve a high degree of functionalization, 10 mL of
41 methacrylic anhydride was added to a 10% w/v gelatin
42 solution in phosphate-buffered saline (PBS) under stirring for
43 1 h. The functionalized polymer was dialyzed against distilled
44 water for 7 days at 40 °C to remove unreacted methacrylic
45 anhydride, freeze-dried and stored at -20 °C protected from
46 light until further use. GelMA constructs were formed by
47 dissolving GelMA in DMEM (including 0.05 % v/v Irgacure
48 (Sigma Aldrich) as photoinitiator) obtaining final
49 concentrations of 5, 10 and 15% w/v. The GelMA solution
50 was poured into polydimethylsiloxane (PDMS) moulds (same
51 dimensions as for the agarose-CaCl₂ moulds) and exposed to
52 UV light for 15 min (365 nm, 5 mW/cm²) to perform the
53 crosslinking. Alginate - GelMA IPN hydrogels were prepared
54 by combining alginate solution (DMEM, 7% w/v) with
55 GelMA solution of various concentrations (DMEM, 10, 20

and 30% w/v) containing 0.05% w/v Irgacure in 1:1 ratio. The
final mixture was poured into the same agarose-CaCl₂ moulds
used for alginate constructs and cured with 45 mM CaCl₂ and
under UV light exposure for 20 and 15 min respectively.

2.2 Isolation and expansion of MSCs and CCs

Bone marrow was removed from the femoral shaft of a
porcine donor and washed in high-glucose Dulbecco's
Modified Eagle Medium (hgDMEM) (Biosciences)
supplemented with 10% foetal bovine serum (FBS), 1%
penicillin (100 U/mL) – streptomycin (100 µg/mL) (all
Bioscience) and amphotericin B (2.5 µg/mL) (Sigma-
Aldrich). A homogenous cell suspension was achieved by
tritulating with a 18G needle. The solution was centrifuged
twice at 650g for 5 min, with removal of the supernatant. The
resultant cell pellet was triturated, and the cell suspension was
filtered through a 40 µm cell sieve (Sarstedt). Cell counting
was performed with trypan blue and acetic acid before plating
at a density of 5 x 10³ cells/cm² and maintained in a humidified
chamber. Following colony formation, cells were trypsinized,
counted and re-plated for a further passage at a density of 5 x
10³ cells/cm². FGF-2 (Prospec-Tany TechnoGene Ltd) was
added to the media which was changed twice weekly.

Cartilage tissue from another porcine donor was rinsed with
PBS, weighed and finely diced. Chondrocytes were isolated
by digestion in hgDMEM containing 1% penicillin (100
U/mL) – streptomycin (100 µg/mL) and 8 mL/g of collagenase
type II (350 U/mL) for 12-14 h under constant rotation at 37
°C. The resulting cell suspension was filtered through a 40 µm
cell sieve, centrifuged and rinsed with PBS twice. Cell number
and viability were determined using a haemocytometer and
0.4% trypan blue staining.

All expansion was conducted in low oxygen conditions
(5% pO₂) and media was changed twice weekly.

2.3 3D bioprinting process

All 3D printed constructs were fabricated using the 3D
Discovery bioplotter purchased from RegenHU with spatial
resolution of ± 5 µm. Porous PCL (CAPA 6500D, Perstorp,
Mn 50 kDa) scaffolds with two different geometries named
Aligned and *Double Offset* were printed using a FDM
printhead. All printing parameters are described in Table 1.

For the *in vitro* evaluation, previously printed PCL
scaffolds were sterilized with ethylene oxide (EtO). Prior to
hydrogels printing, the PCL constructs were placed in each
well of a 6-well plate by means of 3D printed poly lactic acid
inserts containing PDMS moulds that kept the scaffolds in
place in the centre of the well. This allowed for z-direction
printing of the hydrogel bioinks through microextrusion
bioprinting.

Table 1. Summary of FDM and microextrusion printing parameters for PCL and hydrogels.

<i>Printing Parameters</i>	FDM printing	Microextrusion printing		
<i>Material</i>	PCL	3.5% alginate	5% gelMA	IPN
<i>Needle (Gauge)</i>	30	23	23	23
<i>Pressure (bar)</i>	1	0.5	0.3	1.3
<i>Screw Speed (revs/m)</i>	10	-	-	-
<i>Temperature (°C)</i>	86 (tank), 78 (needle)	-	13	13
<i>Layer Thickness (mm)</i>	0.1	-	-	-
<i>Extrusion Speed (mm/s)</i>	6	4	2	4

To form the single hydrogel component bioinks, alginate and GelMA were dissolved at a concentration of 7 and 10 % w/v respectively. To produce the IPN bioink, alginate and GelMA were firstly dissolved to 14 and 20% w/v respectively and then mixed together in 1:1 ratio using a luer lock system (7 and 10% w/v alginate and GelMA final respectively). As co-cultures of BMSCs and CCs have been shown to support stable cartilage tissue formation resistant to hypertrophy and endochondral ossification *in vivo* [64], using the above mentioned luer lock system, the obtained bioinks were then combined with a mixture of BMSCs and CCs (3:1 ratio) in 1:1 ratio (3.5% w/v alginate final, 5% w/v GelMA final, 3.5% w/v alginate – 5% w/v GelMA IPN final, 20 x 10⁶ cells/mL). Cell-encapsulated bioinks were loaded into a syringe and the plunger was moved up and down until air bubbles were collected at the tip of the syringe and could be removed. After minimizing the presence of air bubbles, individual hydrogel components were mixed using a dual syringe approach to form the IPN.

Using a z-direction bioprinting approach, the pores of the previously printed PCL networks were loaded with either alginate, GelMA or alginate-GelMA IPN bioinks. Z-direction bioprinting involved using a generated custom G-code to deposit the required bioink by moving the needle in the z-direction through the open pores of the PCL networks. To make sure that cell-encapsulated bioinks were homogeneously distributed, the PCL networks were soaked in 45 mM CaCl₂ and let rotate overnight before bioprinting. This initiated the crosslinking process of the bioinks as soon as they were extruded, avoiding settling of the hydrogels at the bottom of the PCL scaffolds as well as formation of empty spaces. See Table 2 for printing parameters. Following the fabrication process, the constructs were fully crosslinked as follows: PCL + alginate scaffolds were immersed in a bath of 45 mM CaCl₂

for 20 mins; PCL + GelMA constructs underwent UV light exposure for 15 mins; PCL + IPN constructs were subjected to both CaCl₂ (45 mM) and UV light crosslinking for 15 and 20 min respectively.

2.4 *In vitro* culture conditions

3D bioprinted scaffolds were cultured for 6 weeks in chondrogenically defined media consisting of hgDMEM supplemented with 1% penicillin (100 U/mL) – streptomycin (100 µg/mL), sodium pyruvate (100 µg/mL), L-proline (40 µg/mL), L-ascorbic acid 2-phosphate (50 µg/mL), linoleic acid (4.7 µg/mL), bovine serum albumin (1.5 mg/mL), 1x insulin-transferrin-selenium, dexamethasone (100 nM) (all Sigma-Aldrich) and human TGF-β3 (10 ng/mL) (Prospec-Tany TechnoGene Ltd). Media change was performed twice weekly. To test whether a co-culture of MSCs and CCs within a bioink is capable of promoting the development of phenotypically stable articular cartilage, constructs were maintained at 5% pO₂ for the first 3 weeks of culture to induce robust chondrogenesis, and then switched to 20% pO₂ for the final 3 weeks to test the capacity of the co-culture to maintain a stable cartilage phenotype.

2.5 *Live/Dead confocal microscopy*

Cell viability was evaluated 24 h post-printing using a Live/Dead assay kit (Bioscience). Bioprinted cell-laden scaffolds were rinsed with phenol free medium and incubated in a solution containing 4 µM ethidium homodimer-1 and 2 µM calcein for 1 h. Following incubation, the scaffolds were rinsed again and imaged with Olympus FV-1000 Point-Scanning Confocal Microscope t 488 and 543 nm channels. Cell viability was quantified using Image-J software.

2.6 Biochemical analysis

The biochemical content of all constructs was quantified. Samples were digested with papain (125 µg/mL) in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, 0.05 M ethylene-diamine-tetracetic acid (EDTA) (all Sigma-Aldrich) and pH 6 under constant rotation at 60 °C for 18 h. DNA content was assessed using the Hoechst Bisbenzimidazole 33258 dye assay. The amount of sulphated glycosaminoglycans (sGAG) was measured using the dimethyl methylene blue-binding assay (DMMB) (Blyscan, Bicolor Ltd.). Total collagen content was determined by quantifying the hydroxyproline content using the (dimethylamino)benzaldehyde and chloramine T assay and a hydroxyproline to collagen ratio of 1:7.69. As the gelatin within the GelMA group is derived from collagenous material, day 1 data is not included in relevant figures because the hydroxyproline assay detects the gelatin in the GelMA day 1 samples. Therefore, day 1 data is subtracted from the day 21 and day 42 data for all hydrogel groups to determine the actual produced collagen during *in vitro* culture.

2.7 Histological and Immunohistochemical analysis

Scaffolds were fixed with 4% paraformaldehyde solution, dehydrated in graded series of ethanol, embedded in paraffin wax, sectioned at 8 µm and affixed to microscope slides. The sections were stained with Alcian Blue/Aldehyde Fuchsin, Picrosirius Red and Alizarin Red to assess for sGAG, collagen and calcium content.

Immunohistochemical technique was used to evaluate collagen types II and X. Sections were rehydrated and treated with chondroitinase ABC (Sigma-Aldrich) in a humidified environment at 37 °C to enhance permeability of the extracellular matrix. This was followed by incubation in goat serum to block non-specific sites and the relevant collagen type II (sc52658, 1:400) (mouse monoclonal; Santa Cruz Biotechnology) or collagen type X (ab49945, 1:200) primary antibodies (mouse monoclonal; Abcam) were applied overnight at 4 °C. Treatment with peroxidase preceded the application of the secondary antibody (collagen type II, B7151, 1:300; collagen type X, ab49760, 1:200) at room temperature for 1 h. Thereafter, all sections were incubated with ABC reagent (Vectastain PK-400; Vector Labs) for 45 min. Finally, sections were developed with DAB peroxidase (Vector Labs) for 5 min. Positive and negative controls were included in the immunohistochemical staining protocols.

2.8 Mechanical characterization

All mechanical experiments were performed at room temperature (~25 °C) using a twin column Zwick universal testing machine (Zwick, Roell). Unconfined compression tests were carried out in a PBS bath using a 100 N load cell. To ensure contact between the surface of the scaffolds (n = 4 per group) and the top compression platen, a preload of 0.05 N

was used for hydrogels alone constructs (2 mm high x 6 mm diameter), whereas 0.5 N preload was applied to PCL alone as well as composite constructs (9 mm x 9 mm x 4 mm). Hydrogels alone constructs were produced in the shape of a cylinder (A combined stress-relaxation and dynamic compression protocol (Supplementary Figure 1a) was implemented, where a series of compressive strains were applied in increasing steps of 10% to a maximum of 30%. Peak strain was reached within 500 s and the equilibrium stress was obtained after a relaxation time of 45 min. After the relaxation phase, five compressive cycles at 1% strain at a frequency of 1 Hz were superimposed. Ramp (or compressive), equilibrium and dynamic moduli were quantified at each strain increment. The ramp modulus was calculated as the slope of the initial linear region of the obtained stress-strain curves. The equilibrium modulus was determined as the equilibrium force divided by the sample's cross-sectional area divided by the applied strain. The dynamic modulus was measured as the average force amplitude over the five cycles divided by the sample's cross-sectional area divided by the applied strain amplitude.

Cell-laden 3D bioprinted constructs underwent a shorter version of the stress-relaxation and dynamic compression protocol, to prevent cell death as there was no temperature and/or oxygen control during testing. Samples (n = 3) were subjected to 20% compressive strain followed by 45 min relaxation time and five dynamic compressive cycles at 1% strain and 1 Hz frequency. Ramp, equilibrium and dynamic moduli were quantified as explained in the previous paragraph.

Uniaxial tensile tests on 3D printed dog bone-shaped PCL samples were performed. Each sample (n = 4 per group) was mounted on a sandpaper frame with Araldite glue (Huntsman). The sandpaper frame, along with the PCL scaffold attached to it, was then fixed between two grips. Every test started after cutting through both edges of the sandpaper frame to allow for stretching of the sample. Tensile specimens were tested using a 2500 N load cell at a displacement rate of 1 mm/min and loaded to 50% strain. The tensile modulus was taken as the slope of the stress-strain plots between 2 and 7% strain.

2.9 Finite Element Analysis

IPN only, PCL only and composite constructs (4.5 mm x 4.5 mm x 4 mm) were modelled using axisymmetric FE models developed in Abaqus 6.14 (DS Simulia, USA) to evaluate von Mises stress, maximum principal stress, hydrostatic pressure and scaffold lateral expansion upon loading application. PCL structures were modelled according to a previously described approach [65]. PCL reinforced IPN constructs were simulated by placing an IPN hydrogel matrix inside the pores of the PCL scaffold. A tie interaction between the PCL and IPN meshes was applied in order to constrain the translation degrees of freedom of the common nodes of the

IPN to those of the PCL scaffold. The models simulated ramp compression tests in unconfined configuration, until 10% strain was applied. For all groups, the nodes at the bottom ends of the scaffolds were constrained only in the loading direction, allowing for scaffold lateral expansion in the other two

directions. A displacement of approximately 0.4 mm was prescribed through a platen that was considered a rigid body and in full contact with the nodes at the top surface of the scaffolds. Symmetric boundary conditions were applied to the constructs' inner faces.

Table 2. Material parameters for the PCL and IPN hydrogel used for the numerical analysis.

<i>Material</i>	<i>Material Model</i>	<i>Material Properties</i>
PCL	Isotropic elastic	$E = 430 \text{ MPa}; \nu = 0.3$
	Isotropic plastic	$\sigma_{\text{true}}^y = 17.745 \text{ MPa}; \epsilon_{\text{true}}^y = 0;$ $\sigma_{\text{true}}^f = 113.39 \text{ MPa}; \epsilon_{\text{true}}^f = 1.3316$
IPN	Isotropic elastic	$E = 0.036 \text{ MPa}; \nu = 0.49$

The engineering compressive modulus of the constructs was obtained from the stress and strain values of the linear region of the curve, calculated from the displacement and resultant reaction force data computed from the simulations. Quadratic ten-node tetrahedral elements (C3D10) were used. Material properties were determined from experimental mechanical testing. Isotropic elastic behaviour was assumed for the IPN hydrogel with a Poisson's ratio of 0.49, representing an almost incompressible material [66]. Based on our previous work [65], isotropic elastic and plastic material properties were assigned to the PCL. Material properties of both PCL and IPN are summarized in Table 2.

2.10 Statistical analysis

Results are presented as mean $\pm$ standard deviation. Statistical analysis was performed with GraphPad (GraphPad Software, La Jolla California, USA). Experimental groups were analysed for significant differences using either a t-test or a general linear model for analysis of variance (ANOVA) and performing Tukey's post-test. For all comparisons, significance was accepted for $p \leq 0.05$.

3. Results and discussion

3.1 Alginate - GelMA IPNs possessed superior mechanical properties to their single hydrogel components

The mechanical properties of alginate – GelMA IPN hydrogels were first compared to that of the individual hydrogel components. This was achieved using a series of stress-relaxation tests combined with dynamic tests in an unconfined compression configuration (Supplementary Figure 1a). The ramp phase of the unconfined compression test clearly showed that the IPN hydrogel was significantly stiffer than either the alginate or GelMA hydrogel alone (Supplementary Figure 1b). From the ramp phase of each compression test (to either 10%, 20% or 30% strain), it was possible to quantify the ramp modulus of the hydrogels (Figure 1a; Supplementary Figure 2 a-c; Table 3). The ramp modulus of IPN hydrogel was significantly higher than the alginate and GelMA only hydrogels, and significantly higher than the sum of the individual hydrogel components (shown by the red dashed line) at 20% and 30% strain, demonstrating a synergistic increase in stiffness. Strain stiffening was observed for all three hydrogels, with the ramp modulus increasing with the applied strain amplitude.

Equilibrium and dynamic properties are other important functional parameters to measure when assessing articular cartilage and biomaterials designed to regenerate this tissue [1, 3, 5, 67]. Stress-relaxation tests were next used to determine the equilibrium modulus of the hydrogels in the absence of fluid flow. Stress-time curves (Supplementary Figure 1c) revealed the distinct stress-relaxation behaviour of each hydrogel. Alginate gels exhibited a complete relaxation of the stress after only 900 s, presumably due to the unbinding of the ionic crosslinker followed by polymer matrix flow [68-71]. In contrast, GelMA hydrogels displayed relatively little stress

relaxation. The IPN hydrogel stress-relaxation response fell in between that of the alginate and GelMA alone, where the equilibrium state was reached after about 1500 s.

Both the equilibrium and dynamic modulus of IPN hydrogels were higher than the sum of the individual alginate and GelMA hydrogels, significantly so at 20% and 30% strain (Figure 1b, c; Supplementary Figure 2 d-i; Table 3). The dynamic properties of a biomaterial reflect its capacity to generate fluid load support, which in the case of articular

cartilage is associated with the low permeability of the solid matrix [3, 4].

In spite of the improvements associated with the IPN hydrogel, its equilibrium and dynamic mechanical properties still remained at least one order of magnitude lower than that of native articular cartilage (equilibrium modulus ranges from approximately 0.2 to 2 MPa, while the dynamic modulus ranges from approximately 10 to 60 MPa) [15, 72-74].

Table 3. Summary of constructs mechanical properties under unconfined compression.

	Ramp Modulus			Equilibrium Modulus			Dynamic Modulus		
	(MPa)			(MPa)			(MPa)		
	10% strain	20% strain	30% strain	10% strain	20% strain	30% strain	10% strain	20% strain	30% strain
3.5% Alginate	0.011 ± 0.003	0.044 ± 0.031	0.049 ± 0.026	0.001 ± 0.001	0.006 ± 0.005	0.007 ± 0.007	0.046 ± 0.034	0.066 ± 0.058	0.094 ± 0.095
5% GelMA	0.008 ± 0.001	0.015 ± 0.003	0.018 ± 0.002	0.005 ± 0.002	0.005 ± 0.003	0.007 ± 0.002	0.015 ± 0.006	0.018 ± 0.005	0.027 ± 0.005
IPN	0.036 ± 0.009	0.110 ± 0.035	0.214 ± 0.048	0.013 ± 0.011	0.028 ± 0.005	0.034 ± 0.001	0.147 ± 0.044	0.265 ± 0.070	0.385 ± 0.090
Double Offset PCL	0.221 ± 0.060	0.304 ± 0.041	0.623 ± 0.092	0.246 ± 0.041	0.261 ± 0.043	0.395 ± 0.036	0.515 ± 0.127	0.777 ± 0.117	1.603 ± 0.205
Double Offset PCL + IPN	0.775 ± 0.081	1.034 ± 0.378	1.445 ± 0.256	0.203 ± 0.215	0.652 ± 0.284	0.957 ± 0.527	0.757 ± 0.195	1.415 ± 0.285	2.293 ± 0.352

Figure 1. (a) Ramp modulus, (b) equilibrium modulus and (c) dynamic modulus in unconfined compression of 3.5% alginate (blue bars), 5% GelMA (orange bars) and IPN (black bars) hydrogels when applying increasing levels of strain amplitude: 10,

20 and 30%. The sum of the modulus of the single component hydrogels for each applied strain is indicated by the dashed red line. # $p < 0.01$ and ### $p < 0.001$ IPN vs. 3.5% alginate and 5% GelMA at 20% applied strain; \$\$\$\$ $p < 0.0001$ IPN vs. 3.5% alginate and 5% GelMA at 30% strain amplitude; * $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ vs. IPN; $n = 4$ per group.

3.2 3D printing of PCL networks with tension-compression nonlinearity

Recognizing that hydrogels alone [43, 75-79], even IPN hydrogels, fail to recapitulate the complex mechanical

properties of articular cartilage, we next sought to investigate whether it was possible to design more biomimetic materials with nonlinear mechanical properties by reinforcing IPN hydrogels with 3D printed PCL networks with distinct tension-compression nonlinearity.

Table 4. Summary of compressive and tensile properties for *Aligned* and *Double Offset* PCL.

	Compressive Modulus (MPa)	Tensile Modulus (MPa)
<i>Aligned PCL</i>	1.341 ± 0.095	14.795 ± 0.278
<i>Double Offset PCL</i>	0.218 ± 0.014	8.358 ± 0.990

Figure 2. (a) Schematic of the PCL scaffolds depicting the *Aligned* geometry characterized by a regular orthogonal architecture and the *Double Offset* geometry comprising of offset layers present in both xz and yz planes. Offset layers are shown in blue. Scaffold architecture features are also indicated: h , height of the scaffold; l , length of the scaffold; s , fibre spacing. (b) Microscopy images of *Aligned* (left column) and *Double Offset* (right column) constructs. Scale bar: 2 mm. (c) Tensile modulus, (d) compressive modulus and (e) ratio between tensile and compressive modulus of *Aligned* (black bars) and *Double Offset* (grey bars) 3D printed PCL scaffolds. **** $p < 0.0001$; $n = 4$ per group.

To this end, 3D printing parameters (Table 1) were varied in order to obtain two distinct 3D PCL geometries named *Aligned* and *Double Offset* (Figure 2a, b). The *Aligned* geometry (Figure 2a) was characterized by a regular orthogonal architecture in a lay-down pattern of 0°0°/90°90° forming square pores. The *Double Offset* geometry (Figure 2a) consisted of an orthogonal architecture with the same lay-down pattern. This differed from the *Aligned* pattern because of the presence of offset layers every other four layers that were deposited at an offset distance equal to half the fibre spacing. The obtained 3D network/scaffold architectures were tested in uniaxial tension and compression configurations to evaluate the effect of network geometry on the degree of tension-compression nonlinearity. *Aligned* constructs were stiffer in both tension and compression compared to *Double Offset* networks (Figure 2c, d; Table 4), with more dramatic differences observed in the compressive properties of the PCL networks. The compressive modulus was 1.341 ± 0.095 and 0.218 ± 0.014 MPa for *Aligned* and *Double Offset* samples respectively (Figure 2c; Table 4), whereas the tensile stiffness was 14.795 ± 0.278 MPa for the *Aligned* geometry and 8.358

± 0.990 MPa for the *Double Offset* one (Figure 2d; Table 4). When quantifying the ratio between tensile and compressive moduli, higher tension-compression nonlinearity was observed in the *Double Offset* networks in comparison to *Aligned* samples (Figure 2e).

3.3 Combining IPN hydrogels with reinforcing PCL networks to produce biomimetic composites with mechanical properties comparable to native cartilage

As tension-compression nonlinearity is believed to play a critical role in the load-bearing capacity of articular cartilage by enhancing fluid pressurization under compression, we hypothesized that reinforcing IPN hydrogels with a *Double Offset* PCL network design would result in the development of constructs that more closely mimicked the mechanical behaviour of the native tissue. To test this hypothesis, we next reinforced the alginate – GelMA IPNs with 3D printed PCL networks with two distinct architectures (*Aligned* and *Double Offset*).

Figure 3. (a) Schematic illustration of the mechanical testing set-up and scaffold groups (PCL, IPN and PCL + IPN) that underwent compression tests. (b,c) Ramp modulus of PCL frames (red bars), IPN hydrogels (black bars) and PCL + IPN scaffolds (grey bars) when varying GelMA concentration in the IPN from 5 to 15% (w/v). Results are shown for groups containing either the (b) *Aligned* or (c) *Double Offset* PCL geometry. The sum of the compressive modulus of the individual components for each GelMA concentration is indicated by the dashed blue line. ***p<0.001 and ****p<0.0001; n = 4 per group.

Figure 4. (a) Ramp modulus, (b) equilibrium modulus and (c) dynamic modulus in unconfined compression of IPN hydrogel (black bars), *Double Offset* PCL (red bars) and PCL + IPN (grey bars) scaffolds when applying increasing levels of strain amplitude: 10, 20 and 30%. The sum of the moduli of the individual components for each applied strain is indicated by the dashed blue line. &&&p<0.001 PCL + IPN vs. PCL at 10% applied strain; ##p<0.01 and ####p<0.0001 PCL + IPN vs. PCL at 20% applied strain; \$p<0.05, \$\$\$p<0.001 and \$\$\$\$p<0.0001 PCL + IPN vs. PCL at 30% applied strain; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. PCL + IPN; n = 4 per group.

The PCL scaffolds were infused with different alginate - GelMA IPN hydrogel formulations where the alginate concentration (3.5% w/v) was maintained constant, whereas the GelMA content was increased (5, 10 and 15% w/v). The resulting composite constructs were subjected to compression tests to determine their ramp modulus in comparison to PCL scaffolds alone, IPNs alone and the sum of the moduli of the individual components (Figure 3a-c; Supplementary Figure 3). After infusing the *Double Offset* PCL scaffolds with the IPN hydrogels (Figure 3b), a synergistic increase in stiffness was observed for all IPN formulations. Specifically, the ramp modulus of the composite samples (grey bars) was higher than the sum of the moduli of the single components (blue dashed lines) as well as to the individual components, i.e. the PCL scaffolds (red bars) and IPN gels (black bars). The ramp modulus of the composite increased as the GelMA content in the IPN increased (0.554 ± 0.090 , 0.665 ± 0.140 and 0.713 ± 0.153 MPa at 5, 10 and 15% w/v GelMA concentration, respectively). Moreover, *Double Offset* PCL + IPN constructs were significantly stiffer than the *Double Offset* PCL alone samples (0.218 ± 0.014 MPa), showing approximately 2.5-, 3- and 3.3-fold increases in stiffness at 5, 10 and 15% w/v GelMA concentrations, respectively. The obtained ramp modulus values for the *Double Offset* PCL + IPN constructs fell in the range of native tissue properties ranging from approximately 0.24 to 1.4 MPa [73, 80, 81]. Combining the *Aligned* PCL constructs with the IPN hydrogels (Figure 3c) did not result in the same synergistic increase in stiffness. The ramp moduli of the composite constructs did not increase as the GelMA content in the IPN gels increased (1.147 ± 0.149 , 1.183 ± 0.164 and 1.206 ± 0.195 MPa at 5, 10 and 15% w/v

GelMA concentration, respectively). Furthermore, there was no statistical difference in ramp modulus compared to the *Aligned* PCL alone scaffolds (1.261 ± 0.051 MPa), indicating that the PCL component was dominating the mechanical behaviour of the composite.

The ramp, equilibrium and dynamic moduli of the composites were also assessed after the application of 10, 20 and 30% strain for the *Double Offset* PCL design (Figure 4a-c; Supplementary Figure 4; Table 3). Stiffness was found to increase with increases in the applied strain amplitude. For all strain levels, the ramp moduli was significantly higher for the composite constructs (grey bars; 0.775 ± 0.081 , 1.034 ± 0.378 and 1.445 ± 0.256 MPa when applying 10, 20 and 30% strains respectively) compared to the PCL alone (red bars; 0.221 ± 0.060 , 0.304 ± 0.041 and 0.623 ± 0.092 MPa at 10, 20 and 30% applied strain amplitudes respectively), the IPN alone (black bars) as well as the sum of the individual components (blue dashed line) at all applied strain amplitudes (Figure 4a; Supplementary Figure 4 a-c; Table 3). The equilibrium modulus of the PCL + IPN composites was only higher than the PCL scaffolds alone at higher strain levels.

In unconfined compression, the equilibrium modulus of articular cartilage ranges from approximately 0.2 to 2 MPa [67, 73, 74, 82-84], whereas the dynamic modulus in response to cyclic loading ranges from approximately 10 to 60 MPa, depending on the loading frequency [15, 31, 73, 85, 86]. The composite 3D printed PCL reinforced IPN constructs possessed equilibrium moduli that fell in the range of native cartilage equilibrium properties (Figure 4b; Supplementary Figure 4 d-f; Table 3). Although the dynamic modulus of PCL + IPN composites did not match the values of the native tissue,

it showed a marked improvement compared to PCL and IPN alone (Figure 4c; Supplementary Figure 4 g-i; Table 3), reaching the same order of magnitude as articular cartilage. The dynamic modulus for composite constructs was measured to be 0.757 ± 0.195 , 1.415 ± 0.285 and 2.293 ± 0.352 MPa at 10, 20 and 30% applied strain amplitudes respectively, whereas it increased from 0.515 ± 0.127 to 1.603 ± 0.205 MPa and 0.147 ± 0.044 to 0.385 ± 0.090 MPa for PCL and IPN alone, respectively. The fact that the dynamic properties of the composite did not reach native values is likely related to the fact its permeability is significantly higher than that of

articular cartilage. While a lower permeability (and hence a superior capacity to generate fluid load support) could be achieved by increasing the density of the IPN, this could negatively impact nutrient transport and waste removal once the biomaterial is seeded with cells for tissue engineering applications. Hence, the properties achieved here can be viewed as a balance between providing initial mechanical function (mimicking native equilibrium and ramp modulus values) and an environment supportive of chondrogenesis (that is assessed below).

Figure 5. Computational and experimental (a,b) compressive stress-strain curves and (c,d) ramp modulus for (a,c) *Double Offset* and (b,d) *Aligned* PCL-based structures; ***p<0.001; n = 4 per group. Comparison of predicted IPN (e) lateral expansion

and (f) hydrostatic pressure in IPN alone, *Aligned* PCL+IPN and *Double Offset* PCL+IPN structures. Von Mises stress contour plots for (g) *Double Offset* and (h) *Aligned* PCL-based constructs.

3.4 FE modelling to better understand the mechanism by which 3D printed fibre networks mechanically reinforce IPN hydrogels

FEA was next utilized to better understand the mechanism by which the PCL networks were mechanically reinforcing the IPN hydrogels.

FE simulations were performed of uniaxial compression tests on the IPN alone, PCL alone and composite constructs for both *Double Offset* and *Aligned* PCL-based structures (Figure 5). The predicted stress-strain behaviour (Figure 5a, b) and compressive modulus (Figure 5c, d) of all groups were in good agreement with the experimental results. Having demonstrated that this computational approach was able to accurately predict the compressive mechanical behaviour of 3D printed scaffolds, we next sought to investigate how the *Double Offset* PCL network was reinforcing the IPN hydrogels. When articular cartilage is subjected to compressive loading, the collagen network resists lateral expansion and fluid pressurization supports a significant component of the applied load. When comparing the expansion in both x- and y-direction of IPN in *Double Offset* PCL + IPN, *Aligned* PCL + IPN and IPN alone models, it was observed that reinforcing the IPN with either of the PCL networks reduced lateral expansion of the composite, with the lowest expansion observed in the *Double Offset* PCL composite (Figure 5e). We next predicted the average hydrostatic pressure produced in the IPN when applying 10% compressive strain. In general, higher pressure was generated within the IPN in the presence of both *Aligned* and *Double Offset* PCL, with the highest values predicted in *Double Offset* PCL + IPN structures. Compared to IPN alone, there was approximately a 24- and 7.5-fold increase in hydrostatic pressure in *Double Offset* PCL + IPN and *Aligned* PCL + IPN constructs, respectively (Figure 5f). The FE model also predicted higher stresses in the PCL filaments within the *Double Offset* PCL network in composites compared to *Double Offset* PCL only structures (Figure 5g). On the other side, little difference in von Mises stress distribution was predicted in the PCL network of the *Aligned* PCL + IPN composites compared to *Aligned* PCL only scaffolds (Figure 5h). Together, the FE models suggest two related mechanisms by which the *Double Offset* PCL network reinforces the IPN hydrogel. Firstly, the presence of the IPN in the PCL network increases the tensile stresses generated in the PCL filaments (see also Supplementary Figure 5), allowing them to play a greater role in supporting the applied load. Secondly, the PCL network is predicted to limit lateral expansion of the

composite and lead to higher magnitudes of hydrostatic pressure being generated within the IPN. Such reinforcement mechanisms are enhanced by PCL networks with greater tension-compression nonlinearity.

Other studies have reported similar reinforcement mechanisms, mainly when reinforcing hydrogels with electrospun fibres. For example, Castilho et al. [66] demonstrated that the reinforcement effect of microfibre reinforced GelMA composites derived either from fibres being pulled in tension by the expansion of the hydrogel, or from the support provided by the hydrogel in preventing buckling of the fibres under compression.

The FE simulations further demonstrate that the combination of the *Double Offset* PCL network with the IPN produce a cartilage biomimetic construct.

3.5 PCL reinforced cell-laden IPNs fabricated using 3D bioprinting support chondrogenesis with minimal hypertrophy

Having successfully obtained composite constructs with mechanical properties comparable to those of native cartilage, we proceeded to assess the capacity of these composites to provide a pro-chondrogenic environment *in vitro*. With a view to bioprinting cell-laden implants, we established a multiple-tool biofabrication process that first involved the bioprinting of the porous reinforcing *Double Offset* PCL networks in the shape of a cylinder (6 mm x 6 mm). In a second bioprinting step, the obtained PCL networks were placed in a moulding system that kept them in the centre of the well of a 6-well plate, which facilitated the z-direction bioprinting of the bioinks (alginate, GelMA and alginate - GelMA IPNs containing a co-culture of BMSCs and CCs) into the PCL networks (Figure 6a). Z-direction bioprinting consists of depositing the cell encapsulating-bioinks within the empty pores of the previously printed PCL networks by moving the needle in the z-direction. The bioprinted constructs were then transferred to chondrogenic media and cultured in static conditions at 5% O₂ for the first 3 weeks and then at 21% O₂ for further 3 weeks.

To verify that cell viability was not affected by the 3D printing process or the post-fabrication crosslinking of the bioinks, all constructs were analysed using live-dead staining after 24 h of culture (Figure 6b-c). All bioinks supported high levels of cell viability (~80%; Figure 6c), showing that the fabrication process was not overly harmful to the cells.

Over 6 weeks of *in vitro* culture, the ramp modulus of the PCL reinforced GelMA and IPN-based composites progressively increased (Figure 6d-f; Table 5).

Table 5. Summary of mechanical properties of alginate-, GelMA- and IPN-based composites under unconfined compression after 1, 21 and 42 days of culture.

	Ramp Modulus (MPa)			Equilibrium Modulus (MPa)			Dynamic Modulus (MPa)		
	D1	D21	D42	D1	D21	D42	D1	D21	D42
3.5% Alginate	0.678 ± 0.141	0.822 ± 0.228	0.830 ± 0.114	0.269 ± 0.083	0.318 ± 0.060	0.332 ± 0.063	0.647 ± 0.123	0.773 ± 0.193	0.774 ± 0.211
5% GelMA	0.320 ± 0.094	0.732 ± 0.062	0.927 ± 0.082	0.296 ± 0.062	0.282 ± 0.036	0.362 ± 0.113	0.565 ± 0.088	0.812 ± 0.278	1.174 ± 0.345
IPN	0.926 ± 0.177	1.133 ± 0.015	1.469 ± 0.326	0.235 ± 0.063	0.309 ± 0.078	0.390 ± 0.055	0.727 ± 0.192	0.925 ± 0.190	0.945 ± 0.158

The PCL reinforced-IPN constructs possessed the highest ramp modulus at all time points reaching values of 1.469 ± 0.326 MPa compared to 0.830 ± 0.114 and 0.927 ± 0.082 MPa for alginate-based and GelMA-based composites respectively at day 42 (Figure 6d; Table 5). No major differences in the equilibrium modulus were observed between the different groups, which was approximately 0.350 MPa after 42 days of culture (Figure 6e; Table 5). Significant increases in dynamic modulus with time in culture were only observed in the GelMA-based composites (Figure 6f; Table 5).

All three PCL reinforced bioinks supported the development of a hyaline cartilage-like tissue that stained positive for GAG and collagen type II deposition (Figure 7a-c). GAG and collagen deposition appeared quite pericellular within the IPN-based composites at day 21 (Figure 7b) but become more diffuse with further time in culture (Figure 7c). What was presumed to be non-specific type X collagen staining, was observed in the GelMA-based composites at day 1, making it difficult to assess type X collagen deposition in this group. There was no positive staining for collagen type X in the other groups at any time point, suggesting that the engineered tissues were not becoming hypertrophic with time in culture. Alginate and GelMA-based scaffolds showed some positive staining for alizarin red at day 42, demonstrating the presence of calcium deposits. In contrast, no evidence of calcium deposition was observed in IPN-based composites, suggesting then the development of phenotypically stable articular cartilage-like tissue within the IPNs. The percentage of tissue staining positive for Alizarin Red was also quantified, which demonstrated that the alginate-based constructs supported the highest levels of calcification, while calcific deposits were barely detectable in the IPN group (Figure 6g). Histological observations were confirmed by biochemical analysis, which demonstrated that GAG and collagen content

significantly increased over the culture period, with no dramatic differences observed between the three hydrogel groups (Figure 7e-f).

Together these results demonstrated that alginate – GelMA IPNs were able to support robust chondrogenesis, with levels of ECM deposition comparable to that observed in the individual hydrogels. Importantly, it was shown that the increased mechanical properties achieved with the IPN did not negatively affect its biological functionality. Usually, hydrogels presenting cell binding motifs such as GelMA can facilitate cell-cell and cell-matrix interactions leading more to fibrocartilage-like tissue formation [78, 87-90]. In contrast, alginate is known to be an inert material and should facilitate MSCs to develop a round shape, which is known to support a chondrogenic phenotype [78, 91, 92]. Given that a co-culture of CCs and MSCs is known to support the generation of phenotypically stable articular cartilage [64, 93-96], we did not expect to see dramatic difference in hypertrophy and progression along an endochondral pathway in the three different hydrogel bioink groups. Importantly, the stiffer IPN based composites supported the development of cartilage resistant to calcification after 6 weeks of culture, despite the fact that stiffer hydrogel environments can be more supportive of chondrocyte hypertrophy [37, 97-99].

Although the architecture of the 3D printed PCL networks did not mimic the arrangement of the collagen fibres in the native articular cartilage, the findings of this study show that the internal fibre pattern of such scaffolds can be tailored to ultimately engineer constructs with key mechanical properties (i.e. nonlinearity, equilibrium and dynamic properties) that match or approach those of cartilage prior to any *in vitro* culture, making them potentially suitable for immediate implantation. In addition, designing PCL networks with an offset pattern results in relatively high tension-compression

nonlinearity, which, similarly to the collagen network in the native tissue, limits lateral expansion of the constructs and enhances the hydrostatic pressure within the IPN during compressive loading. Furthermore, the PCL + IPN composites support robust chondrogenesis, ensuring that the mechanical properties of cell-laden constructs will continue to improve (e.g. with time in culture) as the encapsulated cells secrete a cartilage-like ECM.

As the architecture of the collagenous network is known to playing a key role in determining the load-bearing properties

of soft tissues such as articular cartilage [82], future studies could use 3D bioprinting strategies to develop scaffolds that also replicate specific structural characteristics of cartilage. Specifically, 3D bioprinting could potentially enable the production of either polymer networks that mimic the zone-specific arrangement of the collagen fibres in the native tissue, and/or polymeric fibre networks that could direct the alignment of the newly formed matrix to produce engineered tissues that better mimic the zonal collagenous structure of articular cartilage.

Figure 7. (a-c) Histological and immunohistochemical staining of all 3D bioprinted constructs groups after (a) 1, (b) 21 and (c) 42 days of *in vitro* culture. Scale bar: 500 μ m. (d-f) Biochemical analysis of all composites, including (d) DNA content normalized to wet weight (ng/mg), (e) GAG/DNA and (f) collagen/DNA after day 1, 21 and 42 of *in vitro* culture. (g) Quantification of percentage of Alizarin Red staining within all three composite groups at day 1, 21 and 42. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; $n = 3$ per group.

4. Conclusions

This work represents a significant step towards developing biomechanically functional biomaterials for cartilage tissue engineering. The combination of alginate – GelMA IPN hydrogels with appropriately designed 3D printed PCL networks enables the engineering of composites with mechanical properties that are mimetic of normal articular cartilage. Importantly, such composite constructs provide encapsulated cells with an environment conducive to chondrogenesis, resulting in robust production of articular cartilage-like matrix.

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Reinforcing interpenetrating network hydrogels with 3D printed polymer networks to engineer cartilage mimetic composites

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Abstract

Engineering constructs that mimic the complex structure, composition and biomechanics of the articular cartilage represents a promising route to joint regeneration. Such tissue engineering strategies require the development of biomaterials that mimic the mechanical properties of articular cartilage whilst simultaneously providing an environment supportive of chondrogenesis. Here three-dimensional (3D) bioprinting is used to develop polycaprolactone (PCL) fibre networks to mechanically reinforce interpenetrating network (IPN) hydrogels consisting of alginate and gelatin methacryloyl (GelMA). Inspired by the significant tension-compression nonlinearity of the collagen network in articular cartilage, we printed reinforcing PCL networks with different ratios of tensile to compressive modulus. Synergistic increases in compressive modulus were observed when IPN hydrogels were reinforced with PCL networks that were relatively soft in compression and stiff in tension. The resulting composites possessed equilibrium and dynamic mechanical properties that matched or approached that of native articular cartilage. Finite Element (FE) modelling revealed that the reinforcement of IPN hydrogels with specific PCL networks limited radial expansion and increased the hydrostatic pressure generated within the IPN upon the application of compressive loading. Next, multiple-tool biofabrication techniques were used to 3D bioprint PCL reinforced IPN hydrogels laden with a co-culture of bone marrow-derived stromal cells (BMSCs) and chondrocytes (CCs). The bioprinted biomimetic composites were found to support robust chondrogenesis, with encapsulated cells producing hyaline-like cartilage that stained strongly for sGAG and type II collagen deposition, and negatively for type X collagen and calcium deposition. Taken together, these results demonstrate how 3D bioprinting can be used to engineer constructs that are both pro-chondrogenic and biomimetic of the mechanical properties of articular cartilage.

Keywords: articular cartilage, tissue engineering, interpenetrating network hydrogels, polycaprolactone, 3D bioprinting, finite element modelling, biomimetic scaffolds

1. Introduction

Articular cartilage is a thin layer of connective tissue that lines the ends of bones within synovial joints. Under normal physiological conditions, cartilage provides a smooth, lubricated surface for articulation that facilitates load support

and distribution across the joint. Biomechanically it functions as a multiphasic, fibre-reinforced material that has anisotropic, nonlinear and viscoelastic properties [1-3]. The complex load bearing properties of articular cartilage strongly depend on the anisotropic nature of the tissue, consisting primarily of collagen and proteoglycans, and the associated tension-compression nonlinearity [1, 2, 4, 5]. The negatively charged

proteoglycans within articular cartilage generate an osmotic swelling pressure, which is balanced by a pre-stress that is developed in the collagen network [3, 6]. The compressive properties of cartilage typically increase as the proteoglycans content increases, and the loss of proteoglycans has been shown to make the tissue more prone to microdamage during mechanical loading [1, 7-10]. The architecture and organization of the collagen fibres determine the intrinsic tensile stiffness and strength of cartilage [10-12]. The tensile modulus of articular cartilage is significantly higher than the compressive modulus [13, 14]. When cartilage is tested in unconfined compression, this tension-compression nonlinearity enhances fluid pressurization and elevates the dynamic modulus of the tissue [15, 16], as the collagen network restricts lateral expansion of the tissue during compressive loading. The relative importance of the different matrix components on the overall mechanical properties of articular cartilage has been studied by depleting cartilage samples of collagen and/or proteoglycans [2, 9, 10, 17]. For example, the compressive modulus of the proteoglycan-depleted cartilage matrix may be as low as 3 kPa, representing less than 2% of the normal tissue modulus [18]. Therefore, the collagen in articular cartilage can be considered as a fibrillar matrix that primarily sustains tension. Biomaterial strategies that aim to mimic articular cartilage should consider such complexities in their design.

As articular cartilage has poor regenerative capacity, damage to this tissue impairs its mechanical function and can lead to an abnormal loading within the joint. If left untreated, the damage can progress to osteoarthritis of the joint, causing significant pain and disability. This motivates the need for new regenerative approaches capable of restoring the normal biomechanics of the joint.

A number of tissue engineered strategies to engineer cartilage replacements have been developed [19-27]. A typical approach is to design biomaterial environments capable of supporting chondrogenesis; this commonly involves combining cells and/or bioactive molecules with hydrogels as a scaffolding material due to their high water content and attractive transport properties [28-31]. Other than presenting pro-chondrogenic biological cues to the encapsulated cells, such biomaterial scaffolds must also provide a suitable mechanical environment capable of instructing cell fate as well as withstanding the challenging physiological loading conditions experienced *in vivo* upon implantation into the body [27]. Commonly used single network hydrogels (e.g. alginate, GelMA, agarose, polyethylene glycol, etc.) have been extensively studied and have been shown to provide an environment that is conducive to chondrogenesis. However, they are typically mechanically weak and unable to withstand the challenging environment of the joint. Recently, more complex hydrogel systems such as IPN hydrogels, characterized by the combination of multiple polymer

networks have been investigated. Within such IPN hydrogels individual polymer networks are physically entangled with each other and crosslinked only with themselves using specific chemistries [32-35]. In general, such IPN hydrogels have displayed synergistic increases in mechanical properties, including increased toughness and elasticity when compared to the single hydrogel components. However, they do not mimic many of the features of load bearing tissues like articular cartilage, such as anisotropy and tension-compression nonlinearity. Furthermore, engineering IPN hydrogels with bulk mechanical properties approaching that of articular cartilage typically requires very high hydrogel polymer concentrations, resulting in biomaterial environments that are not compatible with robust extracellular matrix (ECM) deposition by encapsulated cells [36-38].

To address these concerns, hydrogels can be reinforced with networks of fibres to produce composites with improved mechanical properties [2, 31, 39-41]. However, these techniques typically involve the use of complex biofabrication strategies (e.g. weaving, melt electrowriting) that are challenging to scale and personalize to specific patient anatomies. In contrast, Fused Deposition Modelling (FDM) can be used to 3D print fibre networks that are relatively simple to scale, with internal and external architectures that are easily defined based on medical imaging [42, 43]. This is achieved using PCL, a synthetic thermoplastic polymer which has been extensively used in the field of 3D bioprinting [44]. Advantages include ease of shaping and manufacture due to its low melting temperature (~ 60°C), biocompatibility, elasticity and mechanical properties [45]. The mechanical properties of PCL scaffolds can also be tuned by modifying the molecular weight and the scaffold architecture [46]. Other synthetic polymers such as poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) are also biocompatible with FDM, with PLGA also allowing for more tailorable degradation rates [47-49]. A concern with the use of PLGA is the release of acidic products as the polymer degrades, which can induce inflammation [50, 51]. Another major disadvantage is the higher melting temperature of PLA and PLGA compared to PCL, which is not ideal when co-printing with live cells.

In this study, alginate – GelMA IPNs were reinforced with PCL networks printed using FDM to develop biomechanically competent composite constructs for cartilage tissue engineering. [30, 52]. Alginate is a widely used biomaterial in tissue engineering because of its excellent biocompatibility, relatively high control over stiffness and capability to form highly porous structures which facilitate the diffusion of large molecules in and out of the hydrogel [53-55]. However, alginate is often limited under mechanical loading due to its brittleness and low elasticity [56-58]. GelMA is a gelatin-based material that has high biocompatibility, degrades in response to matrix metalloproteinases (MMP), possesses

RGD sequences that favour cell adhesion and is highly elastic [59, 60]. GelMA also possesses relatively poor mechanical strength and toughness [59, 61, 62], however IPNs of alginate and GelMA have previously been shown to have superior mechanical properties to their individual hydrogel components and to be supportive of cell growth and differentiation [59]. Here, alginate, GelMA and alginate – GelMA IPN hydrogel scaffolds were first mechanically characterized to investigate the benefit of combining the two single hydrogel components for engineering cartilage-mimetic hydrogels. These hydrogel bioinks were then reinforced with 3D printed PCL networks with architectures and mechanical properties inspired by the collagen network of articular cartilage. The reinforcement mechanism resulting from the combination of PCL networks with IPN hydrogels was examined using Finite Element Analysis (FEA). Finally, the ability of 3D bioprinted cell-laden PCL reinforced IPN constructs to support chondrogenesis was assessed *in vitro* over 6 weeks of culture.

2. Materials and methods

2.1 Materials preparation

Alginate was prepared by dissolving Alginate (PRONOVA UP LVG) in DMEM to make up a final concentration of 3.5% w/v. Cylindrical constructs were obtained by pouring the alginate solution into 2 mm high-6 mm diameter agarose-CaCl₂ moulds (DMEM, 3% w/v agarose, 45 mM CaCl₂) and allowed to crosslink for 20 min at room temperature. GelMA synthesis was previously described [63]. Briefly, GelMA was synthesized by reaction of porcine type A gelatin (Sigma-Aldrich, average molecular weight 40-50 kDa) with methacrylic anhydride (Sigma-Aldrich) at 50 °C for 4 h. To achieve a high degree of functionalization, 10 mL of methacrylic anhydride was added to a 10% w/v gelatin solution in phosphate-buffered saline (PBS) under stirring for 1 h. The functionalized polymer was dialyzed against distilled water for 7 days at 40 °C to remove unreacted methacrylic anhydride, freeze-dried and stored at -20 °C protected from light until further use. GelMA constructs were formed by dissolving GelMA in DMEM (including 0.05 % v/v Irgacure (Sigma Aldrich) as photoinitiator) obtaining final concentrations of 5, 10 and 15% w/v. The GelMA solution was poured into polydimethylsiloxane (PDMS) moulds (same dimensions as for the agarose-CaCl₂ moulds) and exposed to UV light for 15 min (365 nm, 5 mW/cm²) to perform the crosslinking. Alginate - GelMA IPN hydrogels were prepared by combining alginate solution (DMEM, 7% w/v) with GelMA solution of various concentrations (DMEM, 10, 20

and 30% w/v) containing 0.05% w/v Irgacure in 1:1 ratio. The final mixture was poured into the same agarose-CaCl₂ moulds used for alginate constructs and cured with 45 mM CaCl₂ and under UV light exposure for 20 and 15 min respectively.

2.2 Isolation and expansion of MSCs and CCs

Bone marrow was removed from the femoral shaft of a porcine donor and washed in high-glucose Dulbecco's Modified Eagle Medium (hgDMEM) (Biosciences) supplemented with 10% foetal bovine serum (FBS), 1% penicillin (100 U/mL) – streptomycin (100 µg/mL) (all Bioscience) and amphotericin B (2.5 µg/mL) (Sigma-Aldrich). A homogenous cell suspension was achieved by triturating with a 18G needle. The solution was centrifuged twice at 650g for 5 min, with removal of the supernatant. The resultant cell pellet was triturated, and the cell suspension was filtered through a 40 µm cell sieve (Sarstedt). Cell counting was performed with trypan blue and acetic acid before plating at a density of 5 x 10³ cells/cm² and maintained in a humidified chamber. Following colony formation, cells were trypsinized, counted and re-plated for a further passage at a density of 5 x 10³ cells/cm². FGF-2 (Prospec-Tany TechnoGene Ltd) was added to the media which was changed twice weekly.

Cartilage tissue from another porcine donor was rinsed with PBS, weighed and finely diced. Chondrocytes were isolated by digestion in hgDMEM containing 1% penicillin (100 U/mL) – streptomycin (100 µg/mL) and 8 mL/g of collagenase type II (350 U/mL) for 12-14 h under constant rotation at 37 °C. The resulting cell suspension was filtered through a 40 µm cell sieve, centrifuged and rinsed with PBS twice. Cell number and viability were determined using a haemocytometer and 0.4% trypan blue staining.

All expansion was conducted in low oxygen conditions (5% pO₂) and media was changed twice weekly.

2.3 3D bioprinting process

All 3D printed constructs were fabricated using the 3D Discovery bioplotter purchased from RegenHU with spatial resolution of ± 5 µm. Porous PCL (CAPA 6500D, Perstorp, Mn 50 kDa) scaffolds with two different geometries named *Aligned* and *Double Offset* were printed using a FDM printhead. All printing parameters are described in Table 1.

For the *in vitro* evaluation, previously printed PCL scaffolds were sterilized with ethylene oxide (EtO). Prior to hydrogels printing, the PCL constructs were placed in each well of a 6-well plate by means of 3D printed poly lactic acid inserts containing PDMS moulds that kept the scaffolds in place in the centre of the well. This allowed for z-direction printing of the hydrogel bioinks through microextrusion bioprinting.

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Table 1. Summary of FDM and microextrusion printing parameters for PCL and hydrogels.

<i>Printing Parameters</i>	FDM printing	Microextrusion printing		
<i>Material</i>	PCL	3.5% alginate	5% gelMA	IPN
<i>Needle (Gauge)</i>	30	23	23	23
<i>Pressure (bar)</i>	1	0.5	0.3	1.3
<i>Screw Speed (revs/m)</i>	10	-	-	-
<i>Temperature (°C)</i>	86 (tank), 78 (needle)	-	13	13
<i>Layer Thickness (mm)</i>	0.1	-	-	-
<i>Extrusion Speed (mm/s)</i>	6	4	2	4

To form the single hydrogel component bioinks, alginate and GelMA were dissolved at a concentration of 7 and 10 % w/v respectively. To produce the IPN bioink, alginate and GelMA were firstly dissolved to 14 and 20% w/v respectively and then mixed together in 1:1 ratio using a luer lock system (7 and 10% w/v alginate and GelMA final respectively). As co-cultures of BMSCs and CCs have been shown to support stable cartilage tissue formation resistant to hypertrophy and endochondral ossification *in vivo* [64], using the above mentioned luer lock system, the obtained bioinks were then combined with a mixture of BMSCs and CCs (3:1 ratio) in 1:1 ratio (3.5% w/v alginate final, 5% w/v GelMA final, 3.5% w/v alginate – 5% w/v GelMA IPN final, 20 x 10⁶ cells/mL). Cell-encapsulated bioinks were loaded into a syringe and the plunger was moved up and down until air bubbles were collected at the tip of the syringe and could be removed. After minimizing the presence of air bubbles, individual hydrogel components were mixed using a dual syringe approach to form the IPN.

Using a z-direction bioprinting approach, the pores of the previously printed PCL networks were loaded with either alginate, GelMA or alginate-GelMA IPN bioinks. Z-direction bioprinting involved using a generated custom G-code to deposit the required bioink by moving the needle in the z-direction through the open pores of the PCL networks. To make sure that cell-encapsulated bioinks were homogeneously distributed, the PCL networks were soaked in 45 mM CaCl₂ and let rotate overnight before bioprinting. This initiated the crosslinking process of the bioinks as soon as they were extruded, avoiding settling of the hydrogels at the bottom of the PCL scaffolds as well as formation of empty spaces. See Table 2 for printing parameters. Following the fabrication process, the constructs were fully crosslinked as follows: PCL + alginate scaffolds were immersed in a bath of 45 mM CaCl₂

for 20 mins; PCL + GelMA constructs underwent UV light exposure for 15 mins; PCL + IPN constructs were subjected to both CaCl₂ (45 mM) and UV light crosslinking for 15 and 20 min respectively.

2.4 *In vitro culture conditions*

3D bioprinted scaffolds were cultured for 6 weeks in chondrogenically defined media consisting of hgDMEM supplemented with 1% penicillin (100 U/mL) – streptomycin (100 µg/mL), sodium pyruvate (100 µg/mL), L-proline (40 µg/mL), L-ascorbic acid 2-phosphate (50 µg/mL), linoleic acid (4.7 µg/mL), bovine serum albumin (1.5 mg/mL), 1x insulin-transferrin-selenium, dexamethasone (100 nM) (all Sigma-Aldrich) and human TGF-β3 (10 ng/mL) (Prospec-Tany TechnoGene Ltd). Media change was performed twice weekly. To test whether a co-culture of MSCs and CCs within a bioink is capable of promoting the development of phenotypically stable articular cartilage, constructs were maintained at 5% pO₂ for the first 3 weeks of culture to induce robust chondrogenesis, and then switched to 20% pO₂ for the final 3 weeks to test the capacity of the co-culture to maintain a stable cartilage phenotype.

2.5 *Live/Dead confocal microscopy*

Cell viability was evaluated 24 h post-printing using a Live/Dead assay kit (Bioscience). Bioprinted cell-laden scaffolds were rinsed with phenol free medium and incubated in a solution containing 4 µM ethidium homodimer-1 and 2 µM calcein for 1 h. Following incubation, the scaffolds were rinsed again and imaged with Olympus FV-1000 Point-Scanning Confocal Microscope t 488 and 543 nm channels. Cell viability was quantified using Image-J software.

2.6 Biochemical analysis

The biochemical content of all constructs was quantified. Samples were digested with papain (125 µg/mL) in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, 0.05 M ethylenediamine-tetracetic acid (EDTA) (all Sigma-Aldrich) and pH 6 under constant rotation at 60 °C for 18 h. DNA content was assessed using the Hoechst Bisbenzimidazole 33258 dye assay. The amount of sulphated glycosaminoglycans (sGAG) was measured using the dimethyl methylene blue-binding assay (DMMB) (Blyscan, Bicolor Ltd.). Total collagen content was determined by quantifying the hydroxyproline content using the (dimethylamino)benzaldehyde and chloramine T assay and a hydroxyproline to collagen ratio of 1:7.69. **As the gelatin within the GelMA group is derived from collagenous material, day 1 data is not included in relevant figures because the hydroxyproline assay detects the gelatin in the GelMA day 1 samples. Therefore, day 1 data is subtracted from the day 21 and day 42 data for all hydrogel groups to determine the actual produced collagen during *in vitro* culture.**

2.7 Histological and Immunohistochemical analysis

Scaffolds were fixed with 4% paraformaldehyde solution, dehydrated in graded series of ethanol, embedded in paraffin wax, sectioned at 8 µm and affixed to microscope slides. The sections were stained with Alcian Blue/Aldehyde Fuchsin, Picrosirius Red and Alizarin Red to assess for sGAG, collagen and calcium content.

Immunohistochemical technique was used to evaluate collagen types II and X. Sections were rehydrated and treated with chondroitinase ABC (Sigma-Aldrich) in a humidified environment at 37 °C to enhance permeability of the extracellular matrix. This was followed by incubation in goat serum to block non-specific sites and the relevant collagen type II (sc52658, 1:400) (mouse monoclonal; Santa Cruz Biotechnology) or collagen type X (ab49945, 1:200) primary antibodies (mouse monoclonal; Abcam) were applied overnight at 4 °C. Treatment with peroxidase preceded the application of the secondary antibody (collagen type II, B7151, 1:300; collagen type X, ab49760, 1:200) at room temperature for 1 h. Thereafter, all sections were incubated with ABC reagent (Vectastain PK-400; Vector Labs) for 45 min. Finally, sections were developed with DAB peroxidase (Vector Labs) for 5 min. Positive and negative controls were included in the immunohistochemical staining protocols.

2.8 Mechanical characterization

All mechanical experiments were performed at room temperature (~25 °C) using a twin column Zwick universal testing machine (Zwick, Roell). Unconfined compression tests were carried out in a PBS bath using a 100 N load cell. To ensure contact between the surface of the scaffolds (n = 4 per group) and the top compression platen, a preload of 0.05 N

was used for hydrogels alone constructs (2 mm high x 6 mm diameter), whereas 0.5 N preload was applied to PCL alone as well as composite constructs (9 mm x 9 mm x 4 mm). Hydrogels alone constructs were produced in the shape of a cylinder (A combined stress-relaxation and dynamic compression protocol (Supplementary Figure 1a) was implemented, where a series of compressive strains were applied in increasing steps of 10% to a maximum of 30%. Peak strain was reached within 500 s and the equilibrium stress was obtained after a relaxation time of 45 min. After the relaxation phase, five compressive cycles at 1% strain at a frequency of 1 Hz were superimposed. Ramp (or compressive), equilibrium and dynamic moduli were quantified at each strain increment. The ramp modulus was calculated as the slope of the initial linear region of the obtained stress-strain curves. The equilibrium modulus was determined as the equilibrium force divided by the sample's cross-sectional area divided by the applied strain. The dynamic modulus was measured as the average force amplitude over the five cycles divided by the sample's cross-sectional area divided by the applied strain amplitude.

Cell-laden 3D bioprinted constructs underwent a shorter version of the stress-relaxation and dynamic compression protocol, to prevent cell death as there was no temperature and/or oxygen control during testing. Samples (n = 3) were subjected to 20% compressive strain followed by 45 min relaxation time and five dynamic compressive cycles at 1% strain and 1 Hz frequency. Ramp, equilibrium and dynamic moduli were quantified as explained in the previous paragraph.

Uniaxial tensile tests on 3D printed dog bone-shaped PCL samples were performed. Each sample (n = 4 per group) was mounted on a sandpaper frame with Araldite glue (Huntsman). The sandpaper frame, along with the PCL scaffold attached to it, was then fixed between two grips. Every test started after cutting through both edges of the sandpaper frame to allow for stretching of the sample. Tensile specimens were tested using a 2500 N load cell at a displacement rate of 1 mm/min and loaded to 50% strain. The tensile modulus was taken as the slope of the stress-strain plots between 2 and 7% strain.

2.9 Finite Element Analysis

IPN only, PCL only and composite constructs (4.5 mm x 4.5 mm x 4 mm) were modelled using axisymmetric FE models developed in Abaqus 6.14 (DS Simulia, USA) to evaluate von Mises stress, maximum principal stress, hydrostatic pressure and scaffold lateral expansion upon loading application. PCL structures were modelled according to a previously described approach [65]. PCL reinforced IPN constructs were simulated by placing an IPN hydrogel matrix inside the pores of the PCL scaffold. A tie interaction between the PCL and IPN meshes was applied in order to constrain the translation degrees of freedom of the common nodes of the

IPN to those of the PCL scaffold. The models simulated ramp compression tests in unconfined configuration, until 10% strain was applied. For all groups, the nodes at the bottom ends of the scaffolds were constrained only in the loading direction, allowing for scaffold lateral expansion in the other two directions. A displacement of approximately 0.4 mm was prescribed through a platen that was considered a rigid body and in full contact with the nodes at the top surface of the scaffolds. Symmetric boundary conditions were applied to the constructs' inner faces.

Table 2. Material parameters for the PCL and IPN hydrogel used for the numerical analysis.

<i>Material</i>	<i>Material Model</i>	<i>Material Properties</i>
PCL	Isotropic elastic	$E = 430 \text{ MPa}; \nu = 0.3$
	Isotropic plastic	$\sigma_{\text{true}}^y = 17.745 \text{ MPa}; \epsilon_{\text{true}}^y = 0;$ $\sigma_{\text{true}}^f = 113.39 \text{ MPa}; \epsilon_{\text{true}}^f = 1.3316$
IPN	Isotropic elastic	$E = 0.036 \text{ MPa}; \nu = 0.49$

The engineering compressive modulus of the constructs was obtained from the stress and strain values of the linear region of the curve, calculated from the displacement and resultant reaction force data computed from the simulations. Quadratic ten-node tetrahedral elements (C3D10) were used. Material properties were determined from experimental mechanical testing. Isotropic elastic behaviour was assumed for the IPN hydrogel with a Poisson's ratio of 0.49, representing an almost incompressible material [66]. Based on our previous work [65], isotropic elastic and plastic material properties were assigned to the PCL. Material properties of both PCL and IPN are summarized in Table 2.

2.10 Statistical analysis

Results are presented as mean ± standard deviation. Statistical analysis was performed with GraphPad (GraphPad Software, La Jolla California, USA). Experimental groups were analysed for significant differences using either a t-test or a general linear model for analysis of variance (ANOVA) and preforming Tukey's post-test. For all comparisons, significance was accepted for p ≤ 0.05.

3. Results and discussion

3.1 Alginate - GelMA IPNs possessed superior mechanical properties to their single hydrogel components

The mechanical properties of alginate – GelMA IPN hydrogels were first compared to that of the individual hydrogel components. This was achieved using a series of stress-relaxation tests combined with dynamic tests in an unconfined compression configuration (Supplementary Figure 1a). The ramp phase of the unconfined compression test clearly showed that the IPN hydrogel was significantly stiffer than either the alginate or GelMA hydrogel alone (Supplementary Figure 1b). From the ramp phase of each compression test (to either 10%, 20% or 30% strain), it was possible to quantify the ramp modulus of the hydrogels (Figure 1a; Supplementary Figure 2 a-c; Table 3). The ramp modulus of IPN hydrogel was significantly higher than the alginate and GelMA only hydrogels, and significantly higher than the sum of the individual hydrogel components (shown by the red dashed line) at 20% and 30% strain, demonstrating a synergistic increase in stiffness. Strain stiffening was observed for all three hydrogels, with the ramp modulus increasing with the applied strain amplitude.

Equilibrium and dynamic properties are other important functional parameters to measure when assessing articular cartilage and biomaterials designed to regenerate this tissue [1, 3, 5, 67]. Stress-relaxation tests were next used to determine the equilibrium modulus of the hydrogels in the absence of fluid flow. Stress-time curves (Supplementary Figure 1c) revealed the distinct stress-relaxation behaviour of each hydrogel. Alginate gels exhibited a complete relaxation of the stress after only 900 s, presumably due to the unbinding of the ionic crosslinker followed by polymer matrix flow [68-71]. In contrast, GelMA hydrogels displayed relatively little stress

relaxation. The IPN hydrogel stress-relaxation response fell in between that of the alginate and GelMA alone, where the equilibrium state was reached after about 1500 s.

Both the equilibrium and dynamic modulus of IPN hydrogels were higher than the sum of the individual alginate and GelMA hydrogels, significantly so at 20% and 30% strain (Figure 1b, c; Supplementary Figure 2 d-i; Table 3). The dynamic properties of a biomaterial reflect its capacity to generate fluid load support, which in the case of articular

cartilage is associated with the low permeability of the solid matrix [3, 4].

In spite of the improvements associated with the IPN hydrogel, its equilibrium and dynamic mechanical properties still remained at least one order of magnitude lower than that of native articular cartilage (equilibrium modulus ranges from approximately 0.2 to 2 MPa, while the dynamic modulus ranges from approximately 10 to 60 MPa) [15, 72-74].

Table 3. Summary of constructs mechanical properties under unconfined compression.

	Ramp Modulus			Equilibrium Modulus			Dynamic Modulus		
	(MPa)			(MPa)			(MPa)		
	10% strain	20% strain	30% strain	10% strain	20% strain	30% strain	10% strain	20% strain	30% strain
<i>3.5% Alginate</i>	0.011 ± 0.003	0.044 ± 0.031	0.049 ± 0.026	0.001 ± 0.001	0.006 ± 0.005	0.007 ± 0.007	0.046 ± 0.034	0.066 ± 0.058	0.094 ± 0.095
<i>5% GelMA</i>	0.008 ± 0.001	0.015 ± 0.003	0.018 ± 0.002	0.005 ± 0.002	0.005 ± 0.003	0.007 ± 0.002	0.015 ± 0.006	0.018 ± 0.005	0.027 ± 0.005
<i>IPN</i>	0.036 ± 0.009	0.110 ± 0.035	0.214 ± 0.048	0.013 ± 0.011	0.028 ± 0.005	0.034 ± 0.001	0.147 ± 0.044	0.265 ± 0.070	0.385 ± 0.090
<i>Double Offset PCL</i>	0.221 ± 0.060	0.304 ± 0.041	0.623 ± 0.092	0.246 ± 0.041	0.261 ± 0.043	0.395 ± 0.036	0.515 ± 0.127	0.777 ± 0.117	1.603 ± 0.205
<i>Double Offset PCL + IPN</i>	0.775 ± 0.081	1.034 ± 0.378	1.445 ± 0.256	0.203 ± 0.215	0.652 ± 0.284	0.957 ± 0.527	0.757 ± 0.195	1.415 ± 0.285	2.293 ± 0.352

Figure 1. (a) Ramp modulus, (b) equilibrium modulus and (c) dynamic modulus in unconfined compression of 3.5% alginate (blue bars), 5% GelMA (orange bars) and IPN (black bars) hydrogels when applying increasing levels of strain amplitude: 10,

20 and 30%. The sum of the modulus of the single component hydrogels for each applied strain is indicated by the dashed red line. # $p < 0.01$ and ### $p < 0.001$ IPN vs. 3.5% alginate and 5% GelMA at 20% applied strain; \$\$\$\$ $p < 0.0001$ IPN vs. 3.5% alginate and 5% GelMA at 30% strain amplitude; * $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ vs. IPN; $n = 4$ per group.

3.2 3D printing of PCL networks with tension-compression nonlinearity

Recognizing that hydrogels alone [43, 75-79], even IPN hydrogels, fail to recapitulate the complex mechanical properties of articular cartilage, we next sought to investigate whether it was possible to design more biomimetic materials with nonlinear mechanical properties by reinforcing IPN hydrogels with 3D printed PCL networks with distinct tension-compression nonlinearity.

Table 4. Summary of compressive and tensile properties for *Aligned* and *Double Offset* PCL.

	Compressive Modulus (MPa)	Tensile Modulus (MPa)
<i>Aligned PCL</i>	1.341 ± 0.095	14.795 ± 0.278
<i>Double Offset PCL</i>	0.218 ± 0.014	8.358 ± 0.990

Figure 2. (a) Schematic of the PCL scaffolds depicting the *Aligned* geometry characterized by a regular orthogonal architecture and the *Double Offset* geometry comprising of offset layers present in both xz and yz planes. Offset layers are shown in blue. Scaffold architecture features are also indicated: h , height of the scaffold; l , length of the scaffold; s , fibre spacing. (b) Microscopy images of *Aligned* (left column) and *Double Offset* (right column) constructs. Scale bar: 2 mm. (c) Tensile modulus, (d) compressive modulus and (e) ratio between tensile and compressive modulus of *Aligned* (black bars) and *Double Offset* (grey bars) 3D printed PCL scaffolds. **** $p < 0.0001$; $n = 4$ per group.

To this end, 3D printing parameters (Table 1) were varied in order to obtain two distinct 3D PCL geometries named *Aligned* and *Double Offset* (Figure 2a, b). The *Aligned* geometry (Figure 2a) was characterized by a regular orthogonal architecture in a lay-down pattern of $0^\circ/0^\circ/90^\circ/90^\circ$ forming square pores. The *Double Offset* geometry (Figure 2a) consisted of an orthogonal architecture with the same lay-down pattern. This differed from the *Aligned* pattern because of the presence of offset layers every other four layers that were deposited at an offset distance equal to half the fibre spacing. The obtained 3D network/scaffold architectures were tested in uniaxial tension and compression configurations to evaluate the effect of network geometry on the degree of tension-compression nonlinearity. *Aligned* constructs were stiffer in both tension and compression compared to *Double Offset* networks (Figure 2c, d; Table 4), with more dramatic differences observed in the compressive properties of the PCL networks. The compressive modulus was 1.341 ± 0.095 and 0.218 ± 0.014 MPa for *Aligned* and *Double Offset* samples respectively (Figure 2c; Table 4), whereas the tensile stiffness was 14.795 ± 0.278 MPa for the *Aligned* geometry and 8.358

± 0.990 MPa for the *Double Offset* one (Figure 2d; Table 4). When quantifying the ratio between tensile and compressive moduli, higher tension-compression nonlinearity was observed in the *Double Offset* networks in comparison to *Aligned* samples (Figure 2e).

3.3 Combining IPN hydrogels with reinforcing PCL networks to produce biomimetic composites with mechanical properties comparable to native cartilage

As tension-compression nonlinearity is believed to play a critical role in the load-bearing capacity of articular cartilage by enhancing fluid pressurization under compression, we hypothesized that reinforcing IPN hydrogels with a *Double Offset* PCL network design would result in the development of constructs that more closely mimicked the mechanical behaviour of the native tissue. To test this hypothesis, we next reinforced the alginate – GelMA IPNs with 3D printed PCL networks with two distinct architectures (*Aligned* and *Double Offset*).

Figure 3. (a) Schematic illustration of the mechanical testing set-up and scaffold groups (PCL, IPN and PCL + IPN) that underwent compression tests. (b,c) Ramp modulus of PCL frames (red bars), IPN hydrogels (black bars) and PCL + IPN scaffolds (grey bars) when varying GelMA concentration in the IPN from 5 to 15% (w/v). Results are shown for groups containing either the (b) *Aligned* or (c) *Double Offset* PCL geometry. The sum of the compressive modulus of the individual components for each GelMA concentration is indicated by the dashed blue line. ***p<0.001 and ****p<0.0001; n = 4 per group.

Figure 4. (a) Ramp modulus, (b) equilibrium modulus and (c) dynamic modulus in unconfined compression of IPN hydrogel (black bars), *Double Offset* PCL (red bars) and PCL + IPN (grey bars) scaffolds when applying increasing levels of strain amplitude: 10, 20 and 30%. The sum of the moduli of the individual components for each applied strain is indicated by the dashed blue line. &&&p<0.001 PCL + IPN vs. PCL at 10% applied strain; ##p<0.01 and ####p<0.0001 PCL + IPN vs. PCL at 20% applied strain; \$p<0.05, \$\$\$p<0.001 and \$\$\$\$p<0.0001 PCL + IPN vs. PCL at 30% applied strain; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs. PCL + IPN; n = 4 per group.

The PCL scaffolds were infused with different alginate - GelMA IPN hydrogel formulations where the alginate concentration (3.5% w/v) was maintained constant, whereas the GelMA content was increased (5, 10 and 15% w/v). The resulting composite constructs were subjected to compression tests to determine their ramp modulus in comparison to PCL scaffolds alone, IPNs alone and the sum of the moduli of the individual components (Figure 3a-c; Supplementary Figure 3). After infusing the *Double Offset* PCL scaffolds with the IPN hydrogels (Figure 3b), a synergistic increase in stiffness was observed for all IPN formulations. Specifically, the ramp modulus of the composite samples (grey bars) was higher than the sum of the moduli of the single components (blue dashed lines) as well as to the individual components, i.e. the PCL scaffolds (red bars) and IPN gels (black bars). The ramp modulus of the composite increased as the GelMA content in the IPN increased (0.554 ± 0.090 , 0.665 ± 0.140 and 0.713 ± 0.153 MPa at 5, 10 and 15% w/v GelMA concentration, respectively). Moreover, *Double Offset* PCL + IPN constructs were significantly stiffer than the *Double Offset* PCL alone samples (0.218 ± 0.014 MPa), showing approximately 2.5-, 3- and 3.3-fold increases in stiffness at 5, 10 and 15% w/v GelMA concentrations, respectively. The obtained ramp modulus values for the *Double Offset* PCL + IPN constructs fell in the range of native tissue properties ranging from approximately 0.24 to 1.4 MPa [73, 80, 81]. Combining the *Aligned* PCL constructs with the IPN hydrogels (Figure 3c) did not result in the same synergistic increase in stiffness. The ramp moduli of the composite constructs did not increase as the GelMA content in the IPN gels increased (1.147 ± 0.149 , 1.183 ± 0.164 and 1.206 ± 0.195 MPa at 5, 10 and 15% w/v

GelMA concentration, respectively). Furthermore, there was no statistical difference in ramp modulus compared to the *Aligned* PCL alone scaffolds (1.261 ± 0.051 MPa), indicating that the PCL component was dominating the mechanical behaviour of the composite.

The ramp, equilibrium and dynamic moduli of the composites were also assessed after the application of 10, 20 and 30% strain for the *Double Offset* PCL design (Figure 4a-c; Supplementary Figure 4; Table 3). Stiffness was found to increase with increases in the applied strain amplitude. For all strain levels, the ramp moduli was significantly higher for the composite constructs (grey bars; 0.775 ± 0.081 , 1.034 ± 0.378 and 1.445 ± 0.256 MPa when applying 10, 20 and 30% strains respectively) compared to the PCL alone (red bars; 0.221 ± 0.060 , 0.304 ± 0.041 and 0.623 ± 0.092 MPa at 10, 20 and 30% applied strain amplitudes respectively), the IPN alone (black bars) as well as the sum of the individual components (blue dashed line) at all applied strain amplitudes (Figure 4a; Supplementary Figure 4 a-c; Table 3). The equilibrium modulus of the PCL + IPN composites was only higher than the PCL scaffolds alone at higher strain levels.

In unconfined compression, the equilibrium modulus of articular cartilage ranges from approximately 0.2 to 2 MPa [67, 73, 74, 82-84], whereas the dynamic modulus in response to cyclic loading ranges from approximately 10 to 60 MPa, depending on the loading frequency [15, 31, 73, 85, 86]. The composite 3D printed PCL reinforced IPN constructs possessed equilibrium moduli that fell in the range of native cartilage equilibrium properties (Figure 4b; Supplementary Figure 4 d-f; Table 3). Although the dynamic modulus of PCL + IPN composites did not match the values of the native tissue,

it showed a marked improvement compared to PCL and IPN alone (Figure 4c; Supplementary Figure 4 g-i; Table 3), reaching the same order of magnitude as articular cartilage. The dynamic modulus for composite constructs was measured to be 0.757 ± 0.195 , 1.415 ± 0.285 and 2.293 ± 0.352 MPa at 10, 20 and 30% applied strain amplitudes respectively, whereas it increased from 0.515 ± 0.127 to 1.603 ± 0.205 MPa and 0.147 ± 0.044 to 0.385 ± 0.090 MPa for PCL and IPN alone, respectively. The fact that the dynamic properties of the composite did not reach native values is likely related to the fact its permeability is significantly higher than that of

articular cartilage. While a lower permeability (and hence a superior capacity to generate fluid load support) could be achieved by increasing the density of the IPN, this could negatively impact nutrient transport and waste removal once the biomaterial is seeded with cells for tissue engineering applications. Hence, the properties achieved here can be viewed as a balance between providing initial mechanical function (mimicking native equilibrium and ramp modulus values) and an environment supportive of chondrogenesis (that is assessed below).

Figure 5. Computational and experimental (a,b) compressive stress-strain curves and (c,d) ramp modulus for (a,c) *Double Offset* and (b,d) *Aligned* PCL-based structures; *** $p < 0.001$; $n = 4$ per group. Comparison of predicted IPN (e) lateral expansion

and (f) hydrostatic pressure in IPN alone, *Aligned* PCL+IPN and *Double Offset* PCL+IPN structures. Von Mises stress contour plots for (g) *Double Offset* and (h) *Aligned* PCL-based constructs.

3.4 FE modelling to better understand the mechanism by which 3D printed fibre networks mechanically reinforce IPN hydrogels

FEA was next utilized to better understand the mechanism by which the PCL networks were mechanically reinforcing the IPN hydrogels. FE simulations were performed of uniaxial compression tests on the IPN alone, PCL alone and composite constructs for both *Double Offset* and *Aligned* PCL-based structures (Figure 5). The predicted stress-strain behaviour (Figure 5a, b) and compressive modulus (Figure 5c, d) of all groups were in good agreement with the experimental results. Having demonstrated that this computational approach was able to accurately predict the compressive mechanical behaviour of 3D printed scaffolds, we next sought to investigate how the *Double Offset* PCL network was reinforcing the IPN hydrogels. When articular cartilage is subjected to compressive loading, the collagen network resists lateral expansion and fluid pressurization supports a significant component of the applied load. When comparing the expansion in both x- and y-direction of IPN in *Double Offset* PCL + IPN, *Aligned* PCL + IPN and IPN alone models, it was observed that reinforcing the IPN with either of the PCL networks reduced lateral expansion of the composite, with the lowest expansion observed in the *Double Offset* PCL composite (Figure 5e). We next predicted the average hydrostatic pressure produced in the IPN when applying 10% compressive strain. In general, higher pressure was generated within the IPN in the presence of both *Aligned* and *Double Offset* PCL, with the highest values predicted in *Double Offset* PCL + IPN structures. Compared to IPN alone, there was approximately a 24- and 7.5-fold increase in hydrostatic pressure in *Double Offset* PCL + IPN and *Aligned* PCL + IPN constructs, respectively (Figure 5f). The FE model also predicted higher stresses in the PCL filaments within the *Double Offset* PCL network in composites compared to *Double Offset* PCL only structures (Figure 5g). On the other side, little difference in von Mises stress distribution was predicted in the PCL network of the *Aligned* PCL + IPN composites compared to *Aligned* PCL only scaffolds (Figure 5h). Together, the FE models suggest two related mechanisms by which the *Double Offset* PCL network reinforces the IPN hydrogel. Firstly, the presence of the IPN in the PCL network increases the tensile stresses generated in the PCL filaments (see also Supplementary Figure 5), allowing them to play a greater role in supporting the applied load. Secondly, the PCL network is predicted to limit lateral expansion of the

composite and lead to higher magnitudes of hydrostatic pressure being generated within the IPN. Such reinforcement mechanisms are enhanced by PCL networks with greater tension-compression nonlinearity.

Other studies have reported similar reinforcement mechanisms, mainly when reinforcing hydrogels with electrospun fibres. For example, Castilho et al. [66] demonstrated that the reinforcement effect of microfibre reinforced GelMA composites derived either from fibres being pulled in tension by the expansion of the hydrogel, or from the support provided by the hydrogel in preventing buckling of the fibres under compression.

The FE simulations further demonstrate that the combination of the *Double Offset* PCL network with the IPN produce a cartilage biomimetic construct.

3.5 PCL reinforced cell-laden IPNs fabricated using 3D bioprinting support chondrogenesis with minimal hypertrophy

Having successfully obtained composite constructs with mechanical properties comparable to those of native cartilage, we proceeded to assess the capacity of these composites to provide a pro-chondrogenic environment *in vitro*. With a view to bioprinting cell-laden implants, we established a multiple-tool biofabrication process that first involved the bioprinting of the porous reinforcing *Double Offset* PCL networks in the shape of a cylinder (6 mm x 6 mm). In a second bioprinting step, the obtained PCL networks were placed in a moulding system that kept them in the centre of the well of a 6-well plate, which facilitated the z-direction bioprinting of the bioinks (alginate, GelMA and alginate - GelMA IPNs containing a co-culture of BMSCs and CCs) into the PCL networks (Figure 6a). Z-direction bioprinting consists of depositing the cell encapsulating-bioinks within the empty pores of the previously printed PCL networks by moving the needle in the z-direction. The bioprinted constructs were then transferred to chondrogenic media and cultured in static conditions at 5% O₂ for the first 3 weeks and then at 21% O₂ for further 3 weeks.

To verify that cell viability was not affected by the 3D printing process or the post-fabrication crosslinking of the bioinks, all constructs were analysed using live-dead staining after 24 h of culture (Figure 6b-c). All bioinks supported high levels of cell viability (~80%; Figure 6c), showing that the fabrication process was not overly harmful to the cells.

Over 6 weeks of *in vitro* culture, the ramp modulus of the PCL reinforced GelMA and IPN-based composites progressively increased (Figure 6d-f; Table 5).

Figure 6. (a) Schematic of the experimental set-up: *Double Offset* PCL frames were 3D printed *via* FDM and then placed in a moulding system that kept the PCL scaffolds in the centre of each well of a 6-well plate to facilitate microextrusion (z-direction printing) of the cell-laden bioinks. Obtained composite constructs were cultured in chondrogenic media for the first 3 weeks in hypoxia and the following 3 weeks in normoxia. (b) Representative images of Live/Dead staining used to determine the cell viability of 3.5% alginate, 5% GelMA and IPN bioinks. Scale bar: 200 μ m. (c) Quantitative analysis of the cell viability for all of the bioinks. (d) Ramp modulus, (e) equilibrium modulus and (f) dynamic modulus for all composite groups after day 1, 21 and 42 of culture. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; $n = 3$ per group.

Table 5. Summary of mechanical properties of alginate-, GelMA- and IPN-based composites under unconfined compression after 1, 21 and 42 days of culture.

	Ramp Modulus			Equilibrium Modulus			Dynamic Modulus		
	(MPa)			(MPa)			(MPa)		
	D1	D21	D42	D1	D21	D42	D1	D21	D42
3.5% Alginate	0.678 ± 0.141	0.822 ± 0.228	0.830 ± 0.114	0.269 ± 0.083	0.318 ± 0.060	0.332 ± 0.063	0.647 ± 0.123	0.773 ± 0.193	0.774 ± 0.211
5% GelMA	0.320 ± 0.094	0.732 ± 0.062	0.927 ± 0.082	0.296 ± 0.062	0.282 ± 0.036	0.362 ± 0.113	0.565 ± 0.088	0.812 ± 0.278	1.174 ± 0.345
IPN	0.926 ± 0.177	1.133 ± 0.015	1.469 ± 0.326	0.235 ± 0.063	0.309 ± 0.078	0.390 ± 0.055	0.727 ± 0.192	0.925 ± 0.190	0.945 ± 0.158

The PCL reinforced-IPN constructs possessed the highest ramp modulus at all time points reaching values of 1.469 ± 0.326 MPa compared to 0.830 ± 0.114 and 0.927 ± 0.082 MPa for alginate-based and GelMA-based composites respectively at day 42 (Figure 6d; Table 5). No major differences in the equilibrium modulus were observed between the different groups, which was approximately 0.350 MPa after 42 days of culture (Figure 6e; Table 5). Significant increases in dynamic modulus with time in culture were only observed in the GelMA-based composites (Figure 6f; Table 5).

All three PCL reinforced bioinks supported the development of a hyaline cartilage-like tissue that stained positive for GAG and collagen type II deposition (Figure 7a-c). GAG and collagen deposition appeared quite pericellular within the IPN-based composites at day 21 (Figure 7b) but become more diffuse with further time in culture (Figure 7c). What was presumed to be non-specific type X collagen staining, was observed in the GelMA-based composites at day 1, making it difficult to assess type X collagen deposition in this group. There was no positive staining for collagen type X in the other groups at any time point, suggesting that the engineered tissues were not becoming hypertrophic with time in culture. Alginate and GelMA-based scaffolds showed some positive staining for alizarin red at day 42, demonstrating the presence of calcium deposits. In contrast, no evidence of calcium deposition was observed in IPN-based composites, suggesting then the development of phenotypically stable articular cartilage-like tissue within the IPNs. The percentage of tissue staining positive for Alizarin Red was also quantified, which demonstrated that the alginate-based constructs supported the highest levels of calcification, while calcific deposits were barely detectable in the IPN group (Figure 6g). Histological observations were confirmed by biochemical analysis, which demonstrated that GAG and collagen content

significantly increased over the culture period, with no dramatic differences observed between the three hydrogel groups (Figure 7e-f).

Together these results demonstrated that alginate – GelMA IPNs were able to support robust chondrogenesis, with levels of ECM deposition comparable to that observed in the individual hydrogels. Importantly, it was shown that the increased mechanical properties achieved with the IPN did not negatively affect its biological functionality. Usually, hydrogels presenting cell binding motifs such as GelMA can facilitate cell-cell and cell-matrix interactions leading more to fibrocartilage-like tissue formation [78, 87-90]. In contrast, alginate is known to be an inert material and should facilitate MSCs to develop a round shape, which is known to support a chondrogenic phenotype [78, 91, 92]. Given that a co-culture of CCs and MSCs is known to support the generation of phenotypically stable articular cartilage [64, 93-96], we did not expect to see dramatic difference in hypertrophy and progression along an endochondral pathway in the three different hydrogel bioink groups. Importantly, the stiffer IPN based composites supported the development of cartilage resistant to calcification after 6 weeks of culture, despite the fact that stiffer hydrogel environments can be more supportive of chondrocyte hypertrophy [37, 97-99].

Although the architecture of the 3D printed PCL networks did not mimic the arrangement of the collagen fibres in the native articular cartilage, the findings of this study show that the internal fibre pattern of such scaffolds can be tailored to ultimately engineer constructs with key mechanical properties (i.e. nonlinearity, equilibrium and dynamic properties) that match or approach those of cartilage prior to any *in vitro* culture, making them potentially suitable for immediate implantation. In addition, designing PCL networks with an offset pattern results in relatively high tension-compression

nonlinearity, which, similarly to the collagen network in the native tissue, limits lateral expansion of the constructs and enhances the hydrostatic pressure within the IPN during compressive loading. Furthermore, the PCL + IPN composites support robust chondrogenesis, ensuring that the mechanical properties of cell-laden constructs will continue to improve (e.g. with time in culture) as the encapsulated cells secrete a cartilage-like ECM.

As the architecture of the collagenous network is known to playing a key role in determining the load-bearing properties

of soft tissues such as articular cartilage [82], future studies could use 3D bioprinting strategies to develop scaffolds that also replicate specific structural characteristics of cartilage. Specifically, 3D bioprinting could potentially enable the production of either polymer networks that mimic the zone-specific arrangement of the collagen fibres in the native tissue, and/or polymeric fibre networks that could direct the alignment of the newly formed matrix to produce engineered tissues that better mimic the zonal collagenous structure of articular cartilage.

Figure 7. (a-c) Histological and immunohistochemical staining of all 3D bioprinted constructs groups after (a) 1, (b) 21 and (c) 42 days of *in vitro* culture. Scale bar: 500 μ m. (d-f) Biochemical analysis of all composites, including (d) DNA content normalized to wet weight (ng/mg), (e) GAG/DNA and (f) collagen/DNA after day 1, 21 and 42 of *in vitro* culture. (g) Quantification of percentage of Alizarin Red staining within all three composite groups at day 1, 21 and 42. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$; $n = 3$ per group.

4. Conclusions

This work represents a significant step towards developing biomechanically functional biomaterials for cartilage tissue engineering. The combination of alginate – GelMA IPN hydrogels with appropriately designed 3D printed PCL networks enables the engineering of composites with mechanical properties that are mimetic of normal articular cartilage. Importantly, such composite constructs provide encapsulated cells with an environment conducive to chondrogenesis, resulting in robust production of articular cartilage-like matrix.

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