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A comparison of different bioinks for 3D bioprinting of fibrocartilage and hyaline cartilage

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Abstract
Cartilage is a dense connective tissue with limited self-repair capabilities. Mesenchymal stem cell (MSC) laden hydrogels are commonly used for fibrocartilage and articular cartilage tissue engineering, however they typically lack the mechanical integrity for implantation into high load bearing environments. This has led to increased interest in 3D bioprinting of cell laden hydrogel bioinks reinforced with stiffer polymer fibres. The objective of this study was to compare a range of commonly used hydrogel bioinks (agarose, alginate, GelMA and BioINK™) for their printing properties and capacity to support the development of either hyaline cartilage or fibrocartilage in vitro. Each hydrogel was seeded with MSCs, cultured for 28 days in the presence of TGF-β3 and then analysed for markers indicative of differentiation towards either a fibrocartilaginous or hyaline cartilage-like phenotype. Alginate and agarose hydrogels best supported the development of hyaline-like cartilage, as evident by the development of a tissue staining predominantly for type II collagen. In contrast, GelMA and BioINK™ (a PEGMA based hydrogel) supported the development of a more fibrocartilage-like tissue, as evident by the development of a tissue containing both type I and type II collagen. GelMA demonstrated superior printability, generating structures with greater fidelity, followed by the alginate and agarose bioinks. High levels of MSC viability were observed in all bioinks post-printing (~80%). Finally we demonstrate that it is possible to engineer mechanically reinforced hydrogels with high cell viability by co-depositing a hydrogel bioink with polycaprolactone filaments, generating composites with bulk compressive moduli comparable to articular cartilage. This study demonstrates the importance of the choice of bioink when bioprinting different cartilaginous tissues for musculoskeletal applications.

Introduction

Damage to the cartilaginous tissues of synovial joints is a substantial clinical problem as they have a limited intrinsic capacity for self-repair. Cartilage is a dense connective tissue with a highly organised extracellular matrix (ECM) consisting predominately of proteoglycans (GAG) and collagens [1–3]. The hyaline cartilage found on the articulating surface of bones consists primary of type II collagen, whereas fibrocartilaginous tissues such as the meniscus also contain thicker, type I collagen fibres [4–6]. Regenerating these different types of cartilaginous tissues is challenging, and likely requires the engineering of microenvironments conducive to the development of either a hyaline or fibrocartilage phenotype.

Progress has been made in developing cell based therapies for the repair of focal articular cartilage lesions, damaged menisci and the intervertebral disc [7–9]. One such therapy is autologous chondrocyte implantation (ACI), which is used to treat focal articular cartilage defects. The ACI procedure involves the
uses of chondrocytes harvested from a non-load bearing region of a damaged joint. These cells are expanded in vitro and then injected into the defect site beneath a periosteal flap [10, 11]. Later generations of ACI, such as matrix assisted chondrocyte implantation, involves the use of a supporting biomaterial or scaffold [11]. This addressed challenges such as adequately trapping and supporting the cell suspension beneath a periosteal flap [12]. Another limitation associated with the use of primary chondrocytes for joint repair is their limited availability and tendency to dedifferentiate during in vitro expansion [13, 14], motivating the use of mesenchymal stem cells (MSCs) for orthopaedic tissue engineering applications [15, 16]. When implanting MSCs as part of a tissue engineered construct, the supporting biomaterial should ideally provide cues to direct their differentiation towards specific cell types and thereby enable the development of specialised tissues such as articular cartilage or meniscal fibrocartilage.

Hydrogels are water swollen polymers used to extensively for cartilage tissue engineering, and have been shown to support MSC viability, differentiation and cartilage matrix formation in the presence of chondrogenic factors such as transforming growth factor (TGF)-β3 [17–19]. A limitation of using hydrogels is their poor mechanical properties, which may limit their use in high load bearing environments such as meniscal regeneration or the repair of large articular cartilage defects. One potential solution to this problem is to reinforce the hydrogel with stiffer polymeric fibres [20]. For example, dramatic increases in stiffness have been reported by reinforcing gelatin methacrylate (GelMa) hydrogels with melt-electrospun polycaprolactone (PCL) fibres [21]. Other approaches include hybrid printing involving the co-deposition of hydrogels bioinks alongside fused PCL filaments [22–26]. An advantage of such biofabrication strategies is that it allows for the production of large, anatomically accurate constructs [29–31]. These studies motivate the use of 3D printed polymeric structures to mechanically reinforce cell laden hydrogels for musculoskeletal tissue engineering applications.

If bioprinting of MSC laden hydrogel-PCL composites provides constructs with mechanical properties suitable for articular cartilage or meniscus regeneration, the next question is to identify how the hydrogel bioink itself regulates the phenotype of tissues generated within such constructs. To date the field of biofabrication has placed a strong emphasis on the specific rheological properties of bioinks to allow for controlled extrusion, with arguably less of a focus on their inherent impact on cellular phenotype. The aim of this study was to compare various hydrogel bioinks (agarose, alginate, gelatin methacrylamide (GelMa) and a commercially available PEGMA based hydrogel—BioINK™) for the 3D bioprinting of articular cartilage and fibrocartilage. Specifically, we first sought to compare the capacity of these different bioinks to support chondrogenesis of bone marrow derived MSCs. We then compared the printing fidelity of the different bioinks. In the final phase of the study we sought to reinforce these hydrogels with PCL filaments to engineer mechanically reinforced constructs with properties appropriate for load bearing applications.

Materials and methods

Cell isolation and expansion

Bone marrow derived mesenchymal stem cells (BMSCs) were obtained from the femur of a 4 month old porcine donor. The bone marrow was removed from the femoral shaft and washed in high-glucose Dulbecco’s modified eagle medium (hgDMEM, GlutaMAXTM; GIBCO, Biosciences, Ireland) supplemented with 10% fetal bovine serum (GIBCO, Biosciences, Ireland), 1% penicillin (100 U ml⁻¹) streptomycin (100 μg ml⁻¹), (Biosciences, Ireland). A homogenous suspension was achieved by triturating with a needle. The solution was centrifuged twice at 650 g for 5 min, each time the supernatant was removed. The cell pellet was triturated and the cell suspension was filtered through a 40 μm cell sieve. Following colony formation, cells were trypsinized, counted and re-plated for a further passage at a density of 5 × 10⁴ cells cm⁻². All expansion was conducted in normoxic conditions and media was changed twice weekly. Cells were used at the end of passage 2.

Preparation of materials and cell encapsulation

Each gel had a seeding concentration of 20 million BMSC cells ml⁻¹ and the cylindrical constructs were formed as follows; 4% agarose type VII, (Sigma, Ireland) was dissolved in ultra-pure water and autoclaved. It was heated to 80 degrees and allowed to cool to 40 degrees at which point it was combined with a cell suspension of equal volume for a final agarose concentration of 2%. The agarose/cell suspension (2%; 20 million ml⁻¹) was cast in a stainless steel mould, and allowed to cool for 30 min. Solid constructs was removed using a 5 mm biopsy punch. Alginate was prepared by dissolving Alginate (PRO-NOVA UP LVG) in sterile PBS to make up a final concentration of 3.5% alginate. The constructs were formed by combining a cell pellet with 60 mM CaCl₂, using a 16 G needle to form a cell suspension. This suspension was added to the alginate in a 3:7 ratio and homogeneously mixed in two syringes. The alginate/cell/CaCl₂ suspension was poured into a 4% agarose/60 mM CaCl₂ and allowed to cross-link for 30 min at 37 °C. Individual constructs were isolated using a biopsy punch. GelMa was synthesised by reaction of porcine type A gelatin (Sigma Aldrich) with methacrylic anhydride (Sigma Aldrich) at 50 °C for four hours, as previously described7. Methacrylic anhydride was added to a 10% solution of gelatin in
binding assay each hydrogel using the dimethylmethylene blue dye was estimated by quantifying the amount of sGAG in Bisbenzimide 33258 dye assay. Proteoglycan content Alginate was uncrosslinked using 1 M sodium citrate. Aldrich L-cysteine-HCL, 0.05 M EDTA, and pH 6 L-ascorbic acid 2-phosphate, 4.7 sodium pyruvate, 40 penicillin (cell pellet with the hydrogels. The constructs were formed by combing a freeze-dried and stored at 4°C. PBS under constant stirring. To achieve a high degree of functionalization, 0.6 g of methacrylic anhydride was added per gram of gelatin. The functionalized polymer was dialyzed against distilled water for 7 days at 40°C to remove methacrylic acid and anhydride, freeze-dried and stored at −20°C until use. NMR was used to confirm functionalisation of the GelMA hydrogels. The constructs were formed by combing a cell pellet with the final concentration of GelMa 10% (Irgacure 2959 0.05%). The suspension was poured into a mould and subjected to UV light for 15 min to complete the cross-linking. Solid constructs was removed using a 5mm biopsy punch. BioINK™ is a commercially available PEGMA hydrogel (RegenHU, Switzerland) did not require any further modification. A cell pellet was combined directly with the BioINK™ using a 16 G needle to triturate and exposed to UV for 15 min. Once again the constructs were fabricated using a biopsy punch. The gels were cultured for 4 weeks in chondrogenically defined media consisting of DMEM GlutaMAX supplemented with 100 U ml⁻¹ penicillin/streptomycin (both Gibco), 100 μg ml⁻¹ sodium pyruvate, 40 μg ml⁻¹ L-proline, 50 μg ml⁻¹ L-ascorbic acid 2-phosphate, 4.7 μg ml⁻¹ linoleic acid, 1.5 mg ml⁻¹ bovine serum albumin, 1x insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma–Aldrich) and 10 ng ml⁻¹ human TGF-β3 (Prospec-Tany TechnoGene Ltd, Israel). Media exchange was performed twice weekly.

Biochemical analysis
The biochemical content of constructs (n = 3–4) was assessed. Samples were digested with papain (125 mg ml⁻¹) in 0.1 M sodium acetate, 5 mM L-cysteine-HCL, 0.05 M EDTA, and pH 6 (all Sigma-Aldrich) under constant rotation at 60°C for 18 h. Alginate was uncrosslinked using 1 M sodium citrate. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay. Proteoglycan content was estimated by quantifying the amount of sGAG in each hydrogel using the dimethylmethylen blue dye binding assay (Blyscan, Biocolor Ltd).

Histological and immunohistochemical evaluation
Two samples per group were fixed in 4% paraformaldehyde (Sigma-Aldrich), dehydrated with a graded series of alcohol, and embedded in paraffin. Eight micrometre sections were produced of the cross section of construct face. Sections were stained with Aldehyde Fuschin and 1% alcian blue 8GX (Sigma-Aldrich) in 0.1 M HCL for sGAG accumulation. Collagen types I, II, and X were evaluated using a standard immunohistochemical technique; sections were rehydrated and treated with chondroitinase ABC (Sigma-Aldrich) in a humidified environment at 37°C to enhance permeability of the ECM. This was followed by incubation in goat serum to black non-specific sites and the relevant primary collagen type I (ab90395, 1:400) or collagen type II (ab3092, 1:100) or collagen type X (ab49945, 1:100) primary antibodies (mouse monoclonal; Abcam) were applied overnight at 4°C. Treatment with peroxidase preceded the application of the secondary antibody (collagen type I and II, B7151, 1:5:200; 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.

Mechanical testing
Mechanical tests were performed using a single column Zwick (Zwick, Roell, Germany) with a 100 KN load cell as previously described [32]. Briefly, stress relaxation tests were performed using impermeable metal platen. The equilibrium modulus was determined by applying a 10% unconfined compressive strain with a ramp displacement of 0.001 mm s⁻¹. A relaxation period of 30 min was used.

Live/dead cell assay
Cell viability was established using a live/dead assay kit (Invitrogen, Bioscience). Printed cell-laden constructs were incubated in XPAN media for 30 min prior to the assay. All constructs were rinsed in PBS and incubated for 1 h in a solution containing 2 μM calcein and 4 μM ethidium homodimer-1. After incubation the constructs 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.

3D bioprinting process
A 3D bioplotter from RegenHU (3D Discovery) was used for printing. Polycaprolactone (PCL), Mw = 45 000, (Sigma-Aldrich) was melted at 70°C in the printing chamber. A screw driven piston (25 rev/min, screw diameter 1 cm) extruded the PCL onto a coverslip at a pressure of 0.45 MPa. Parameters for printing of the individual hydrogels are described in the results section. A straight needle was used throughout. A 25 Gauge needle was used to plot PCL resulting in fibre diameters of approximately 250 μm.

Statistics
Statistical analyses were performed using GraphPad Prism. One way ANOVA was used for analysis of variance with Bonferroni post-tests to compare between groups. Numerical and graphical results are displayed as mean ± standard deviation. Significance was accepted at a level of p < 0.05.
Results and discussion

Hydrogel bioinks for cartilage and fibrocartilage tissue engineering

The composition of the cell laden constructs was found to depend on the hydrogel within which they were encapsulated. Histological and immunohistochemical staining at the end of the 4 week culture period demonstrated that the different hydrogels could support the synthesis of either hyaline or fibrocartilage-like tissue components. MSC laden alginate hydrogels stained intensely for sGAG and collagen type II (figure 1(A)). Agarose hydrogels also stained intensely for collagen type II, however sGAG staining was weaker compared to alginate (figure 1(A)). In contrast, GelMA and PEGMA (BioINK™) hydrogels stained weaker for sGAG and collagen type II, with stronger staining for collagen type I (figure 1(A)). Biological analysis confirmed the histological findings, with alginate supporting the highest and GelMA supporting the lowest levels of GAG synthesis (figure 1(B)). There were no significant differences in sGAG accumulation within the different hydrogels when normalised to construct wet weight (figure 1(D)) due to differences in swelling over the 4 week culture period.

Together these results suggest that the MSC laden alginate and agarose hydrogels support the development of more hyaline cartilage-like phenotype, while the GelMA and PEGMA based hydrogels supported the development of a more fibrocartilage-like phenotype. The cells in articular cartilage and meniscus are morphologically and phenotypically distinct, with the rounded chondrocytes in articular cartilage producing predominantly collagen type II, while the fibrochondrocytes found in meniscal and other fibrocartilaginous tissue produce higher levels of collagen type I [33]. Fibrochondrocytes also produce lower levels of sGAG compared to articular chondrocytes [34]. Neither the agarose or alginate present natural cell binding motifs, facilitating the development of a round MSC shape which is known to support a chondrogenic phenotype [35, 36]. In contrast, the GelMA hydrogels present natural cell binding motifs that can support cell spreading [37, 38] and other cell–matrix interactions that may facilitate the development of a more fibrocartilaginous phenotype [39, 40]. The commercial PEGMA based bioink contains type I collagen, again likely contributing to the development of more fibrocartilage-like matrix components, as type I hydrogels have previously been shown to promote high levels of collagen type I mRNA expression in MSCs [41]. Previous studies would suggest that hydrogels need to be functionalised with type II collagen or other cartilage ECM components in order to enhance chondrogenesis of MSCs [42, 43]. Matrix stiffness has been shown to influence the chondrogenic capacity of MSCs, with softer matrices shown to support higher levels of chondrogenesis [44, 45]. For example MSCs cultured in soft hyaluronic acid hydrogels (3.5 kPa) supported higher levels of sGAG and collagen production compared to those cultured in stiffer matrices (<20 kPa) [46]. This may also partially explain why the softer alginate hydrogels (see figure 4A) were supporting more robust chondrogenesis.

Comparison of hydrogel printability

We next evaluated the printability of each hydrogel. The filament spreading ratio, defined as the width of the printed filament divided by the needle diameter, was measured for each bioink. Lower spreading ratios are desirable to allow the fabrication of cell laden hydrogel structures with high precision. A detailed description of the hydrogel printing properties can be found in table 1, including the printing parameters adopted for each hydrogel. GelMA possessed the lowest spreading ratio (1.43 ± 0.36) and importantly was more consistent (as evident by the lowest standard deviation) compared to all other hydrogels (figure 2). Alginate (3.35 ± 0.93) and agarose (2.38 ± 1.06) had higher spreading ratios with higher variability among the filaments. The PEGMA based bioink was the least printable hydrogel (6.06 ± 1.60) making it difficult to recapitulate a prescribed 3D pattern (figure 2A).

Evaluation of post-printing cell viability

Cell viability during 3D bioprinting is dependent on the shear stress experienced during extrusion, which in turn is dependent on the viscosity of the solution, the applied pressure and the needle diameter [20]. In addition, any post-printing bioink cross-linking may also impact on cell viability. Since the solution viscosity, applied pressure and post-cross linking mechanism varied across our chosen hydrogels we next evaluated cell viability immediately post-printing for each of the hydrogel bioinks. All bioinks supported high levels of cell viability, with no differences found between the groups (figures 3A and B). When PCL filaments were deposited adjacent to the MSC laden hydrogels cell viability was lower, likely due to increased temperatures generated in the bioinks by the molten PCL, however it remained above 70% in all groups (figure 3C). This indicated that mechanically reinforced MSC laden structures with high cell viability could be 3D bioprinted using each hydrogel. The levels of cell viability presented here are in agreement with the literature for similar printing systems [25, 47, 48]. One possible way to improve cell viability would be to incorporate a conical needle as this has been shown to reduce levels of shear stress cells are exposed to during extrusion [47].

Development of PCL reinforced hydrogels for cartilage tissue engineering

The equilibrium unconfined compressive modulus of the articular (hyaline) cartilage ranges from approximately 0.2–2 MPa [49–51], increasing with depth
through the tissue, while that of meniscal fibrocartilage can range from approximately 0.1–0.3 MPa [52–57]. Within the deeper regions of articular cartilage, the equilibrium compressive modulus can exceed 10 MPa [50]. The equilibrium modulus of all our bioinks fell below that of native articular cartilage and meniscal tissue (figure 4(a)). Reinforcing the bioinks with PCL microfibers (3.5 mm spacing) increased the moduli (544 fold for alginate, 45 fold for GelMA) to within the range of articular cartilage (figure 4(b)). GelMA had a significantly higher equilibrium modulus compared to all other candidate bioinks (figure 4(a)). Although these mechanical properties are above that of native meniscal tissue, by varying the fibre spacing, fibre diameter and polymer molecular
Table 1. Bioprinting processing parameters for each bioink.

<table>
<thead>
<tr>
<th></th>
<th>Alginate</th>
<th>Agarose</th>
<th>PEGMA (BioINK™)</th>
<th>GelMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Printing temperature</td>
<td>21°</td>
<td>37°</td>
<td>21°</td>
<td>28°</td>
</tr>
<tr>
<td>Polymer concentration</td>
<td>3.5% Alginate, 60 mM CaCl$_2$ (Mixed 7:3)</td>
<td>2%</td>
<td></td>
<td>10% GelMa, 0.05% Irgacure</td>
</tr>
<tr>
<td>Post cross-linking mechanism</td>
<td>Calcium chloride 50 mM bath (15 min)</td>
<td>Physical (Temperature) 15 min</td>
<td>UV Light 15 min</td>
<td>UV Light 15 min</td>
</tr>
<tr>
<td>Extrusion pressure</td>
<td>0.2 MPa</td>
<td>0.2 MPa</td>
<td>0.14 MPa</td>
<td>0.06 MPa</td>
</tr>
</tbody>
</table>
weight it is possible to reduce the mechanical properties [58].

Tissue engineering mechanically functional cartilage grafts remains a significant challenge in the field. Despite improvements in chondrogenic culture conditions [59, 60], scaffold and biomaterial design [61, 62] and the introduction of mechanical loading regimes [63, 64] to enhance the development of tissue engineering cartilage, the mechanical properties of such constructs still fall below those of joint tissues such as the meniscus and articular cartilage. In the context of 3D bioprinting, more cartilage-like mechanical properties can be attained by increasing the polymer concentration and cross linking density of hydrogels [62], or through the use of interpenetrating network hydrogels [65], however the use of such dense bioinks can compromise biological functionality. Cells benefit from lower polymer concentrations allowing space for proliferation, differentiation and matrix synthesis [66]. In particular, softer substrates have been shown to promote chondrogenic differentiation of MSCs, with stiffer substrates shown to promote...
terminal hypertrophic differentiation [46]. By decoupling mechanical and biological function using multiple tool biofabrication it is possible to engineer a softer chondrogenic micro-environment surrounded by a network of stiffer reinforcing fibres that can support bulk loading during articulation. Future improvements could include the integration of more advanced biofabrication techniques such as direct melt electrospinning [21, 67] with bioinks optimised for supporting specific cell phenotypes for engineering more biomimetic soft tissues.

Conclusion

Bioinks are often chosen for their printing fidelity, however the results of this study demonstrate that they also have a potent effect on the phenotype of encapsulated cells. Alginate and agarose bioinks were found to support the development of a hyaline-like cartilage tissues, while the GelMA and PEGMA based bioinks better supported the development of a fibrocartilaginous tissue. We also demonstrate the possibility of engineering reinforced composite hydrogels with bulk mechanical properties in the range of human articular and fibro-cartilage. In doing do we demonstrate how MSC laden constructs can be tailored for specific applications by tailoring both the PCL network, to control construct mechanical properties, and the bioink in order to modulate the cells substrate and hence their phenotype.

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Authors disclosure statement

No competing financial interests exist.

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