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Published in:
Acta Biomaterialia

DOI:
10.1016/j.actbio.2013.10.005

Publication date:
2014

Document Version
Peer reviewed version

Link to publication in Heriot-Watt University Research Portal

Citation for published version (APA):
A biomimetic extracellular matrix for cartilage tissue engineering centered on photo-curable gelatin, hyaluronic acid and chondroitin sulfate

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Abstract

The development of hydrogels tailored for cartilage tissue engineering has been a research and clinical goal for over a decade. Directing cells towards a chondrogenic phenotype and promoting new matrix formation are significant challenges that must be overcome for the successful application of hydrogels in cartilage tissue therapies. Gelatin-methacrylamide (Gel-MA) hydrogels have shown promise for the repair of some tissues, but they have not been extensively investigated for cartilage tissue engineering. We encapsulated human chondrocytes in gel-MA based hydrogels, and show that with the incorporation of small quantities of photo-crosslinkable hyaluronic acid methacrylate (HA-MA), and to a lesser extent chondroitin sulfate methacrylate (CS-MA), chondrogenesis and mechanical properties can be enhanced. The addition of HA-MA to Gel-MA constructs resulted in more rounded cell morphologies, enhanced chondrogenesis as assessed by gene expression and immunofluorescence, and increased quantity and distribution of the newly synthesised ECM throughout the construct. Consequently, while the compressive moduli of control Gel-MA constructs increased by 26 kPa after 8 weeks culture, constructs with HA-MA and CS-MA increased by 96 kPa. The enhanced chondrogenic differentiation, distribution of ECM, and improved mechanical properties make these materials potential candidates for cartilage tissue engineering applications.

Keywords: cartilage tissue engineering, hydrogels, photopolymerisation, gelatin, hyaluronic acid, zonal chondrocytes
Introduction

Articular cartilage is a collagen and proteoglycan rich tissue. More specifically though, collagen type II accounts for over 90% of total collagen in the mature tissue [1], and together with proteoglycans, the large quantity of collagen type II provides the tissue with the mechanical properties required to absorb impacts and withstand significant loads. In the superficial zone of cartilage, lubricating molecules such as proteoglycan 4 (PRG4) allow for low friction movement in articulating joints. However, cartilage is frequently damaged through trauma or disease, and has little to no capacity for self-repair. Without intervention, damaged cartilage continues to degenerate to the point where pain and impaired mobility result, and a joint replacement is typically required. Cartilage tissue engineering is a promising approach to repair damaged cartilage, thereby alleviating the prolonged degeneration and ultimate joint replacement, and to this end, regenerative medicine approaches for cartilage repair have been widely studied and tested for over two decades [2, 3].

One of the major limitations of regenerative cartilage therapies has been the inability to regenerate tissue with the original composition and structure of articular cartilage. In approximately 50% of cases, the repair tissue from autologous chondrocyte implantation (ACI) [2] resembles fibrocartilage and is rich in collagen type I, instead of collagen type II [4, 5]. This is most likely due to chondrocyte dedifferentiation during expansion in two-dimensional cultures. Consequently, the repair tissue often lacks the biomechanical properties required to persist in a loaded joint. These inconsistent ACI results have prevented ACI procedures from becoming the routine first line of treatment for cartilage defects [4]. Thus, for cartilage tissue engineering to be ultimately successful, it will be critical to control the phenotype of chondrocytes, and the composition and organisation of the extracellular matrix (ECM) they produce [6]. The design of biomaterials to promote chondrogenic differentiation
and guide cartilage ECM synthesis is a key approach to improve cartilage tissue engineering outcomes, and has attracted significant research interest in the past decade [7-10]. Various materials have also been tested in next-generation ACI procedures, in which cells are seeded onto biomaterials prior to implantation [11]. Gelatin-methacrylamide (Gel-MA) hydrogels have attracted attention for tissue engineering applications in recent years [12], however their potential to be applied in cartilage tissue engineering has not been widely investigated.

Gelatin is produced by the hydrolysis of ECM-derived collagens, predominantly collagen type I. Gel-MA can be produced by chemical modification of gelatin, allowing hydrogels to be covalently crosslinked in the presence of a photoinitiator and light [13]. During hydrolysis and chemical modification, some conformational structures are irreversibly altered, but Gel-MA hydrogels retain some properties of collagens and gelatin, such as cell adhesion domains [12], thermosensitivity [13] and enzymatic degradability [14]. Gel-MA hydrogels are gaining popularity as biomaterials, since they support the formation of new ECM, are enzymatically degradable, can be produced at low cost, are potentially injectable or pintable, are easily crosslinked under physiological conditions, and show potential for cartilage tissue engineering [15].

In this study, we aimed to enhance chondrocyte behaviour in Gel-MA-based hydrogels by incorporating glycosaminoglycans (GAGs) into the hydrogels. Two of the most abundant GAGs in cartilage are hyaluronic acid (HA) and chondroitin sulfate (CS), and we incorporated these into Gel-MA hydrogels separately or together. We used methacrylated HA and CS (HA-MA and CS-MA, respectively) to allow covalent and stable incorporation of these GAGs into the Gel-MA hydrogels. Table 1 shows the composition and notation used for the hydrogel groups tested in this study.

Materials and Methods

Macromer synthesis
Gelatin (porcine skin, type A), chondroitin sulfate A and methacrylic anhydride (MAAh) were purchased from Sigma Aldrich (St Louis, Mo, USA). Hyaluronic acid (molecular weight 0.86 MDa) was generously provided by Novozymes. Gel-MA, HA-MA and CS-MA were synthesised by reaction of gelatin, HA and CS respectively with MAAh, using protocols based on published methods [13, 16]. Briefly, gelatin, HA and CS were dissolved in phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA) at 10%, 1% and 25% w/v, respectively. Gelatin was reacted with 0.6 g of MAAh per gram of gelatin for 1 hour at 50 °C [13]. CS and HA were reacted with MAAh for 24 hours on ice, with the pH regularly adjusted to 8 with sodium hydroxide [16, 17]. For HA and CS the molar excess of MAAh over the hydroxyl groups was 5- and 10-fold, respectively. After the reaction period, insoluble MAAh was removed by centrifugation, followed by dialysis against deionised water to remove remaining unreacted MAAh and methacrylic acid. The pH of the dialysed polymer solutions was adjusted to 7.4, then they were freeze-dried and stored at -20 °C.

**Cell isolation and expansion**

The procedure for isolating zonal chondrocytes from cartilage has been described in detail elsewhere [18]. Briefly, zonal cartilage samples were excised from the macroscopically normal regions of the femoral condyle of a patient undergoing knee replacement surgery, with consent and ethics approval from the Prince Charles Hospital and Queensland University of Technology [18]. Chondrocytes were isolated and expanded in low glucose DMEM with 2 mM glutamax (Invitrogen), supplemented with 10% fetal bovine serum (Lonza, Waverly Australia), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1 mM non-essential amino acids, 0.5 μg/mL amphotericin B (Fungizone®), 50 U/mL penicillin G sodium, 50 μg/mL streptomycin (all Invitrogen), 0.4 mM L-proline and 0.1 mM ascorbic acid (both Sigma).

**Cell encapsulation and culture**
All hydrogel precursor solutions were prepared in PBS, and contained 0.05% (w/v) of the photoinitiator Irgacure 2959 (BASF, Ludwigshafen, Germany). Passage 1 chondrocytes were suspended in one of the four precursor solutions (Table 1) at a density of 7 million cells/mL. Hydrogels were crosslinked by 15 minutes exposure to 365 nm light at an intensity of 2.6 mW/cm² in a CL-1000 crosslinker (UVP, Upland, CA, USA). The hydrogels were formed in a custom manufactured teflon mold covered by a glass slide, which produces gels with dimensions of $4 \times 4 \times 2$ mm. The cell-hydrogel constructs were cultured for up to 8 weeks in defined chondrogenic differentiation media (high glucose DMEM with 2 mM glutamax (Invitrogen), 10 mM HEPES, 0.1 mM non-essential amino acids, ITS-G (100× dilution), 0.5 μg/mL amphotericin B (Fungizone®), 50 U/mL penicillin G sodium, 50 μg/mL streptomycin (all Invitrogen), 1.25 mg/mL bovine serum albumin (BSA), 0.4 mM L-proline, 0.1 μM ascorbic acid, 0.1 μM dexamethasone (all Sigma) and 10 ng/mL TGF-β3 (GroPep, Adelaide, SA, Australia)). Cell-free hydrogels were cultured in high glucose DMEM with 1.25 mg/mL BSA, 0.5 μg/mL amphotericin B, 50 U/mL penicillin G sodium and 50 μg/mL streptomycin.

**Viability assay**

Live and dead cells were visualised with fluorescein diacetate (FDA) and propidium iodide (PI, both Sigma), respectively, at day 1, week 5 and week 8. Hydrogel constructs were washed in PBS, incubated in a solution of 5 μg/mL FDA and 10 μg/mL PI for 5 minutes at 37 °C, then washed twice in PBS. Images were captured using a Nikon Eclipse fluorescence microscope.

**Biochemical analyses**

At each media change, the amount of GAGs secreted from cell-free and cell-laden constructs into the culture media was measured using the DMMB assay (pH 1.5) [19]. Absorbances at 525 and 595 nm were measured, and the concentrations were calculated using
the ratio of absorbances, compared to a quadratic standard curve prepared from chondroitin sulfate C (Sigma). Data from the first media change were excluded to allow for diffusion of unbound CS-MA from some gels groups. To quantify the retained GAGs and DNA, cultured constructs were weighed, frozen and freeze-dried. Dried constructs were digested overnight in 1 mg/mL hyaluronidase (Sigma) at 37 °C, followed by overnight digestion with 0.5 mg/mL proteinase K (Invitrogen) at 56 °C. GAG concentration in the digests was measured in the same way as media. For both GAGs secreted and retained, amounts from cell-free gels were subtracted from amounts from cell-laden constructs to determine the amount of GAGs produced by the cells. DNA concentration in the digests was measured using the Quant-iT™ PicoGreen® dsDNA quantification assay (Invitrogen).

**Physical properties**

The compressive moduli of constructs were measured in an unconfined arrangement using an Instron 5848 microtester with a 5 N load cell. During testing constructs were submerged in PBS at 37 °C. A displacement rate of 0.01 mm/s was applied using a non-porous indenter, and the modulus taken from the linear region of the stress-strain curve from 10 – 15% strain [15].

**Immunofluorescence**

Constructs cultured for 8 weeks were frozen in Optimal Cutting Temperature compound (OCT, Sakura, Finetek, Tokyo, Japan) and sectioned. Sections were fixed by 10 minutes incubation with ice-cold acetone, followed by antigen retrieval with 0.1% hyaluronidase (Sigma) for 30 minutes. Sections were blocked with 2% BSA in PBS for 1 hour. Primary antibodies were diluted in 2% BSA in PBS, and applied overnight at 4 °C. Antibodies for collagen type II (II-II6B3, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA, 1:200 dilution), collagen type I (I-8H5, MP Biomedicals, Seven Hills, Australia, 1:300 dilution), aggrecan (MA75A95, Abcam/Sapphire Bioscience, Waterloo,
Australia, 1:5 dilution) and CD44 (H4C4, DSHB, 1:200 dilution) were used. Cell membranes were visualised with CellMask™ Orange plasma membrane stain (Invitrogen, 1:400 dilution). A mouse IgG isotype control antibody (Jackson ImmunoResearch, West Grove, PA, USA, 1:1000 dilution) and secondary antibody only were used as negative controls. Sections were washed twice for 5 minutes in PBS. The goat anti-mouse secondary antibody (Alexa Fluor® 488, Invitrogen, 1:400 dilution) was diluted in 2% BSA in PBS containing 5 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Invitrogen), and applied in the dark for 1 hour. After rinsing and drying, sections were mounted with Prolong Gold (Invitrogen) and imaged using a Zeiss Axio microscope.

To quantify fluorescence intensities in the inter-territorial areas (minimum distance of 50 μm from cell nuclei), five regions (each 50 × 50 pixels) were selected from each of 4 different histological sections. The mean greyscale intensity of each region was calculated using ImageJ software (National Institutes of Health, USA). The circularity of cells was quantified by tracing only well defined cell membranes (n > 30) shown in CD44 immunofluorescence images using ImageJ.

**Gene expression**

Total RNA was isolated from freshly trypsinised cells and from hydrogel constructs that were cultured for 5 weeks. Cells or hydrogel constructs were homogenised in 1 mL of TRIzol reagent (Invitrogen), and RNA was isolated according to manufacturer’s instructions. SuperScript™ III First Strand Synthesis System (Invitrogen) was used to synthesise complementary DNA (cDNA). DNase and RNase digestions were performed before and after cDNA synthesis, respectively. Real time-polymerase chain reaction was performed using SybrGreen® Mastermix (Applied Biosystems/Invitrogen). Expression of all genes was normalised to the housekeeping gene RPL13A [20]. The primer sequences (5’ → 3’) used for PCR were as follows: RPL13A [23]: F: CATAGGAAGCTGGGAGCAAG, R:

**EPIC-μCT**

The distribution of fixed negative charges in the constructs was visualised using equilibrium partitioning of an ionic contrast agent microcomputed tomography (EPIC-μCT) [21]. Constructs were incubated in a mixture of 40% (v/v) ioxaglate (Hexabrix, Aspen, Australia) in PBS at 37 °C overnight on a moving plate. Constructs were imaged in a μCT 40 scanner (Scanco Medical, Brüttisellen, Switzerland) at 45 kV and analyzed using Scanco μCT software.

**Statistical analyses**

Statistical analyses were performed using SPSS software (version 20, IBM corporation, USA). Differences between groups were determined using analysis of variance and Tukey’s or Dunnet’s T3 post hoc tests as appropriate, with a significance level of 0.05. Statistically significant differences are indicated in Figures using Roman numerals or symbols. Any two groups labelled with the same Roman numeral are statistically similar, and groups without a like numeral are statistically different.

**Results**
Chondrocytes from the superficial and middle/deep zones responded to the different hydrogels in a similar manner. Thus, data from experiments using chondrocytes from the middle/deep zone, which represents the bulk of the cartilage volume, are presented here.

**Cell numbers, viability and morphology**

High cell viabilities (80-90%) were observed in all constructs after 1 day, 5 weeks and 8 weeks culture, with no differences between groups. Images of live and dead chondrocytes after 8 weeks culture are shown in Fig. 1A-D. The DNA content in all groups increased between day 1 and week 8 (Fig. 1I, all p<0.05), although relatively modestly, with less than 2-fold increases in each group.

The cell membrane receptor CD44 was stained with an antibody to visualise the morphology of the encapsulated cells after 8 weeks culture (Fig. 1E-H), and the average circularity values of at least 30 cells per condition are shown in Fig. 1J. In Gel-MA and G-CS constructs, cell morphologies varied between irregular, stretched morphologies and rounded morphologies (Fig. 1E, G). In gels containing HA-MA, cells had more rounded morphologies (Fig. 1F, H), and the average circularity was higher in these constructs (Fig. 1J). Similarly, the viability images in Fig. 1 show that cells had more rounded morphologies with HA-MA. Although HA-MA influenced the morphology of encapsulated cells, all cells at the surface of all constructs had highly stretched morphologies, as shown by membrane staining (Fig. S1).

**Matrix production and accumulation**

Gel-MA-based hydrogels supported the deposition of new matrix by chondrocytes, however the composition and distribution of the cell-secreted matrix varied between hydrogels. Strong collagen type II and aggrecan immunoreactivity was observed in all gel constructs, however in Gel-MA and G-CS constructs, strong staining was only observed in the regions close to cells, with very little immunoreactivity in the regions between cells (Fig. 2A, C, E, G). In gels containing HA-MA, collagen type II and aggrecan were more
homogenously distributed (Fig. 2B, D, F, H). The average fluorescence intensities of collagen type II and aggrecan in the inter-territorial regions were higher in constructs with HA-MA than those without (Fig. 2 M, N).

In Gel-MA and G-CS constructs, collagen type I antibodies reacted strongly (Fig. 2I, K). The intensity of collagen type I staining was markedly lower in gels with HA-MA (Fig. 2J, L), and the intensity in the inter-territorial regions was also lower with HA-MA (Fig. 2O). In sections treated with only the secondary antibody, the fluorescence intensities of inter-territorial regions were similar for all hydrogel formulations (data not shown). In addition, in constructs with HA-MA, inter-territorial intensities were higher for collagen type II and aggrecan, yet were lower for collagen type I, letting us conclude that differences were due to differential binding of the primary antibodies, rather than background or non-specific interactions. In all constructs, collagen type I staining was strong at the outer surface (Fig. S1).

**GAG production**

Fig. 3 shows GAG production after 8 weeks culture, with all values corrected using cell-free gels. GAG content prior to correction with cell-free gels is shown in Fig. S2, and GAG secretion profiles are shown in Fig. S3. The amount of GAGs retained and secreted (both normalised to wet weight), was higher in G-HA constructs compared to Gel-MA (Fig. 3A). The fraction of the total GAGs that were retained was 60-70% in all constructs, and was not influenced by the presence of HA-MA or CS-MA. When normalised to DNA content, the amount of GAGs retained was also higher in G-HA constructs compared to Gel-MA (Fig. 3B).

**EPIC – μCT**

Cell-free and chondrocyte laden constructs were scanned using EPIC – μCT [25] after 2, 5 and 8 weeks culture, using the negatively charged contrast agent ioxaglate. The intensity
of the ioxaglate signal is inversely proportional to the concentration of groups with a fixed negative charge in the constructs. Fig. 4 shows the scans of Gel-MA and G-HA constructs with middle/deep cells at 2, 5 and 8 weeks, and cell-free constructs at 2 weeks (images from all groups and time points are shown in Fig. S4-6). Attenuation levels in cell-free Gel-MA constructs are higher than in G-HA constructs (Fig. 4 A, E), due to the repulsion of ioxaglate by the negative charges of HA-MA. With increasing culture time, the difference in attenuation levels between Gel-MA and G-HA constructs becomes more pronounced, as a result of enhanced GAG synthesis and deposition in G-HA constructs. Thus EPIC – µCT is a viable tool to visualise differences in fixed negative charges between different cell-free hydrogels, and matrix accumulation in tissue-engineered constructs.

**Gene expression**

The expression levels of chondrogenic marker genes were highly dependent on the hydrogel composition, with greater chondrogenesis occurring in constructs containing HA-MA (Fig. 5). COL2A1 expression was 9-fold higher in G-HA constructs compared to Gel-MA (Fig. 5A). Addition of CS-MA to Gel-MA had a smaller (3-fold increase), but significant effect on COL2A1 expression. ACAN expression was upregulated by the addition of either HA-MA or CS-MA, or both, compared to Gel-MA alone (Fig. 5B), while COL1A1, a dedifferentiation marker, was expressed at lower levels in gels with HA-MA (Fig 5C). Expression of the superficial zone marker PRG4 was higher in gels containing HA-MA (Fig. 5D). PRG4 expression in G-CS gels was intermediate, and statistically similar to both the Gel-MA controls and constructs containing HA-MA. The addition of HA-MA, CS-MA or both lowered the expression of COL10A1 compared to Gel-MA controls (Fig. 5E). Expression of the hypertrophy marker MMP13 was lower in gels containing HA-MA, and expression in these gels was unchanged from day 0 (Fig. 5F). CS-MA had no influence of
MMP13 expression compared to Gel-MA controls, and expression was upregulated in these groups compared to day 0.

Physical properties

Gel-MA hydrogels are sensitive to MMP degradation, yet significant gel degradation was not observed in either the cell-free or cell-laden constructs. Hydrogel constructs were easy to handle at each time point, and swelling after crosslinking was negligible.

The addition of HA-MA increased the stiffness of cell-free gels, and cell-laden gels on day 1 by approximately 10 – 15 kPa, while the addition of CS-MA had no effect on day 1 (Fig. 6A, B). However, HA-MA had a much greater impact on the change in stiffness of cell-laden hydrogels after 8 weeks culture (Fig. 6A, B). After 8 weeks culture, the moduli of Gel-MA constructs increased by 26 kPa, whereas G-HA constructs increased by 96 kPa (Fig. 6B). The addition of CS-MA had a smaller, but significant effect on stiffness, with G-CS constructs increasing by 47 kPa. The largest change in the stiffness was seen in G-HA-CS constructs, which increased by 114 kPa during 8 weeks culture.

HA-MA also had an impact on the changes in the wet weights and swelling ratios of constructs during culture (Fig. 6C, D; Fig. S4). The increases in the wet weights of G-HA and G-HA-CS were lower than those of Gel-MA and G-CS constructs. Similarly, during culture, the swelling ratio of gel-MA constructs increased by more than G-HA and G-HA-CS constructs, while the increase in swelling ratio of G-CS constructs was similar to Gel-MA. Thus after 8 weeks, Gel-MA and G-CS constructs tended to be softer and more highly swollen than those containing HA-MA.

Discussion

HA and CS are abundant GAGs in cartilage ECM, and they have been incorporated into hydrogels and scaffolds with the intention of creating a biomimetic environment to
enhance chondrogenesis [22]. However, there is no clear consensus on the effect that incorporation of GAGs in hydrogels has on chondrogenic differentiation and matrix production. Gelatin-methacrylamide (gel-MA), derived from collagen type I, was the main component in each of the hydrogels used in this study, accounting for at least 90% of the polymer content by mass. This study shows that when used alone, gel-MA does not support complete chondrogenesis in vitro. However with the addition of small quantities of HA-MA, and to a lesser extent CS-MA, gel-MA based hydrogels can provide an instructive environment for the deposition of cartilage-like matrix.

HA is implicated in a vast number of biological processes, including cell proliferation, attachment, migration, differentiation, and tissue homeostasis [23, 24]. The influence of HA on chondrogenesis appears to be dose-dependent, with several studies showing low concentrations of HA to promote chondrogenesis, while higher concentrations have no effect or even inhibit chondrogenesis [25-28]. Despite these complications, HA incorporation into hydrogels has promoted chondrogenesis in several promising studies [29-31], and it remains a key material in cartilage tissue engineering research and clinical application. In this study, gene expression, protein deposition and cell morphology collectively demonstrated that HA-MA significantly enhanced chondrogenesis in the predominantly Gel-MA hydrogels tested.

The benefits of HA-MA were exhibited in three ways: promoting the chondrocyte phenotype; increasing matrix accumulation; and enhancing matrix distribution.

Chondrocytes are highly differentiated cells with a distinct phenotype and cell morphology. The rounded morphology observed in healthy cartilage appears to be intrinsically linked to cell phenotype [32]. When placed on 2-D surfaces coated with ECM molecules, sub-populations of rounded and spread cells can be observed [33]. While rounded cells continue producing predominantly collagen type II, spread cells produce collagen type I
and fibronectin [33], suggesting that reacquiring a rounded morphology may be a requirement for redifferentiation.

HA has been shown to play a critical role in cell attachment to the surrounding matrix [23, 34, 35]. During mitosis and cell migration, cells secrete a thin layer of an HA-rich matrix that mediates detachment and cell rounding [35]. In cartilage, CD44 cell receptors bind HA, which results in the chondrocytes being surrounded by a gel-like layer [36]. Removing this layer rapidly alters the homeostasis of the tissue [36], indicating that HA is critical for regulation of biosynthesis and MMP production. In this study, inclusion of HA-MA supported the rounded cell morphology, and morphology correlated well with other measures of chondrogenic differentiation. We hypothesize that the enhanced chondrocyte morphology in constructs containing HA-MA may be the result of an intrinsic mixing incompatibility between the two polymers. Mixtures of gelatin and HA are cloudy, and, if left to settle in a solution state, eventually phase separate. The phase separation phenomenon may make cell adhesion sites on gelatin less available to cells, resulting in the rounded morphologies.

In G-HA constructs, the main chondrogenic marker, COL2A1, was upregulated 9-fold, whereas the main dedifferentiation marker, COL1A1, was downregulated compared to Gel-MA constructs. While collagen type I staining was markedly reduced in constructs containing HA-MA, two cell subpopulations with different phenotypes were observed. Encapsulated cells had a predominantly rounded morphology and produced only low amounts of collagen type I, while cells at the surface had a fibroblast-like morphology, and secreted high levels of collagen type I. Fibroblastic morphologies and high levels of collagen type I were observed at the surface of all constructs, regardless of hydrogel composition (not shown). Thus at the surface, which is effectively a curved 2-D environment, chondrocytes adopt a dedifferentiated phenotype and produce collagen type I, as they do when cultured on tissue culture plastic [37]. This in vitro result is particularly relevant when compared to some
current clinical practices, such as matrix-induced autologous chondrocyte implantation (MACI®), in which chondrocytes are seeded onto a collagen scaffold [38]. These cells attach to the scaffold, thus effectively being at a surface, rather than encapsulated in a 3-D environment. This may partly account for the limited success of cartilage tissue engineering attempts to date, in which predominantly fibrous cartilage, rather than the desired articular cartilage, is regenerated in the joint.

Incorporation of HA-MA resulted in increased accumulation of cartilage matrix molecules and increased compressive modulus. In addition to higher expression of cartilage-specific genes COL2A1 and ACAN, total GAG production and the GAG/DNA ratio were higher in constructs with HA-MA, indicating that HA-MA promoted increased biosynthesis of cartilage specific matrix molecules. HA interacts with components of the ECM, particularly aggrecan and hyaladherins [39], which may play a role in matrix retention [31]. Of the total amount of GAGs produced during 8 weeks of culture, 60-70% were retained in all constructs, with no significant effect of HA-MA (or CS-MA) on GAG retention.

Mechanical properties are a critical metric to assess the quality of tissue-engineered cartilage. Cartilage from bovine femoral condyles has a Young’s modulus of approximately 0.3 MPa [40], and by attracting water, GAGs account significantly for the compressive stiffness of the tissue [41]. However in this study, higher GAG levels did not fully account for changes in mechanical properties. After 8 weeks culture, differences in compressive moduli were much more pronounced than differences in GAG content. For example, the concentration of GAGs in G-HA constructs was 43% higher than in Gel-MA constructs, while, the increase in compressive moduli of G-HA constructs was 270% greater than the increase in gel-MA constructs.

An explanation for the discrepancy in construct modulus and GAG content may lie in the third beneficial effect of HA-MA: enhancing matrix distribution. In addition to GAGs, the
collagen network is also expected to contribute to the stiffness of the constructs, so GAG content alone is not always an accurate predictor of mechanical properties. HA-MA enhanced the distribution of collagen type II and aggrecan, shown by the higher levels of these components in the inter-territorial regions of constructs with HA-MA. As a result, the mechanical properties of constructs with HA-MA increased significantly more during culture than those without. The newly synthesised matrix is expected to be more interconnected in constructs with HA-MA, and hence provide greater a mechanical reinforcement.

The mechanisms by which HA-MA enhanced the distribution of new matrix and improved the mechanical properties are not clear, but we hypothesise that the micro-scale phase separation of gel-MA and HA-MA helped facilitate distribution of the ECM. In a previous study, in which hydrogels were prepared from either Gel-MA or HA-MA, we found that collagen type II and aggrecan were highly localised to the pericellular regions of HA-MA, suggesting that HA-MA alone does not facilitate larger scale matrix distribution [42]. Similarly, other studies have shown that HA-MA hydrogels alone do not necessarily promote matrix distribution, but lower crosslink densities, and hence larger pore sizes, do allow for more matrix diffusion [43].

Both the hydrogels and cell-secreted ECM are sensitive to enzymatic degradation [44], so the changes in mechanical properties of the constructs during culture are a result of the balance between anabolic and catabolic factors, combined with hydrolytic degradation. The compressive moduli of all cell laden constructs increased during culture, whereas cell-free constructs decreased slightly or were unchanged. The increase in stiffness of cell-laden constructs can therefore be attributed to the matrix deposited by the cells, suggesting that in all gels, regardless of composition, there was a net shift in favour of anabolic processes.

Constructs with HA-MA expressed lower levels of MMP13 than those without. MMP13, also called collagenase-3, is a marker of chondrocyte hypertrophy [45, 46], and is
up-regulated in osteoarthritis, where it is a key mediator of cartilage degradation [47]. In this study, constructs with elevated MMP13 expression levels were also softer and more swollen, thus cell-mediated degradation of both the gelatin, as well as the newly produced ECM, may be a key factor limiting matrix accumulation in these constructs. Exposure to collagen types I and II can upregulate MMP13 production via integrins or discoidin domain receptor 2 (DDR2) [48, 49], but exposure to denatured collagens and gelatin do not have the same effect [48]. Thus, although the cause of MMP13 upregulation in Gel-MA and G-CS constructs is unknown, it could be a result of the high levels of collagen type I that are produced in these gels.

The initial mechanical properties of hydrogels are dependent on the crosslink density, and hence pore size, of the hydrogel, and as expected, larger pore sizes facilitate more diffusion of newly secreted matrix [43, 50, 51]. Therefore, hydrogels that are initially the softest can become the stiffest after a period of in vitro culture, while hydrogels that are initially very stiff may impede the formation and redistribution of new ECM [43, 51]. Interestingly, in this study the opposite trend was observed, with the stiffness increasing by more in the gels that were initially stiffer, showing that HA-MA was facilitating matrix organisation by other means. When optimising the initial crosslink density for the purposes of cartilage repair, it is important to consider both the initial and developed mechanical properties, along with the overall strategy for cartilage repair.

The incorporation of CS-MA had fewer and less significant effects on chondrocytes and constructs properties than HA-MA. This could be because CS does not participate in cell-matrix interactions to the same extent as HA. It could also be because mixtures of gelatin and CS-MA are fully miscible, thus if the phase separation phenomenon observed for HA-MA was indeed important, it would be expected that chondrocytes would respond differently to CS-MA and HA-MA. The solubility limit of CS is substantially higher than that of HA, so
much higher concentrations of CS-MA could potentially be incorporated into gel-MA hydrogels. Thus although the effects of CS-MA were comparatively minor at 0.5%, higher concentrations may elicit stronger chondrocyte responses, and further studies should evaluate the concentration-dependent influence of CS-MA.

The incorporation of CS-MA increased COL2A1 and ACAN gene expression compared to Gel-MA controls, however COL1A1 and MMP13 expression levels were unchanged. Previous studies using PEG-based hydrogels have shown CS-MA to have variable effects on bovine chondrocytes. In one study, CS-MA was shown to strongly upregulate the expression of collagen type II and aggrecan [52], while in another study, CS-MA reduced proteoglycan synthesis by bovine chondrocytes by 22 – 50%, depending on CS-

When both HA-MA and CS-MA were included in the hydrogel construct, cells showed a similar response to those in G-HA constructs, indicating that HA-MA was a more potent modulator of differentiation and physical properties than CS-MA. The greatest increase in stiffness was seen in G-HA-CS constructs, suggesting that there may be advantages including both GAGs in the hydrogels.

Mesenchymal precursor cells (MPCs) have been widely investigated as a potential cell source for the repair of cartilage defects. Since G-HA hydrogels promote the chondrogenic redifferentiation of expanded chondrocytes, further studies could evaluate the potential for these hydrogels to be used to guide chondrogenic differentiation of MSCs. In addition, further studies should validate whether the in vitro results shown here can be translated to enhanced quality of repair tissue in vivo.

Conclusions

This study highlights the potential for multiple-component photo-crosslinkable hydrogels based primarily on Gel-MA to be used in cartilage tissue engineering.
Encapsulated chondrocytes remain viable for up to 8 weeks in culture, and produce a significant amount of ECM. Incorporation of a relatively small proportion of HA-MA into these hydrogels significantly enhances chondrogenesis and facilitates matrix distribution, with corresponding improvements to mechanical properties. Incorporation of CS-MA enhances some aspects of chondrocyte redifferentiation, however to a lesser extent than HA-MA.

Acknowledgements
The research leading to these results has received funding from the European Union’s Seventh Framework Programme (FP7/2007-2013) under grant agreement n°309962 (project HydroZONES). In addition, the authors would like to thank the Australian Research Council, the European Union (Marie Curie Fellowship PIOF-GA-2010-272286 to Melchels), and the Dutch Arthritis Foundation (Malda) for funding. They are also grateful to Prof. Ross Crawford for obtaining cartilage tissue. The antibodies against collagen type II and CD44 were obtained from the Developmental Studies Hybridoma Bank, which is maintained by the University of Iowa, Department of Biology, Iowa City, IA, 52242.

References


Appendix. Supplementary data
**Fig. 1.** Viability (A-D) and morphology (E-H) of chondrocytes cultured for 8 weeks. Total DNA content (I) after 1 day, 5 weeks, and 8 weeks culture, and circularity of cells after 8 weeks culture (J). In live/dead images, living cells appear green and dead cells appear red. In cell morphology images, immunoreactive regions to the membrane receptor CD44 are shown in green, and nuclei were counterstained with DAPI, shown in blue. Scale bars: 100 μm. For DNA quantification, bars and error bars show the mean and standard deviation of 4 samples, and # indicates that within each group, DNA content is different to the value at day 1 (p < 0.05). In J, groups without a like Roman numeral are statistically different (p < 0.05).
Fig. 2. Extracellular matrix production after 8 weeks culture. Immunoreactive regions for collagen type II (A-D), aggrecan (E-H) and collagen type I (I-L) appear green, and nuclei were counterstained with DAPI (blue). The fluorescence intensities in the regions between cells (inter-territorial regions) were quantified for collagen type II (M), aggrecan (N) and collagen type I (O). Scale bars: 100 μm. Box plots show data from a total of 20 regions from 4 different histological sections. Groups without a common Roman numeral are statistically different (p < 0.05).
**Fig. 3.** GAG synthesis in hydrogel constructs after 8 weeks culture, with all values corrected using cell-free gels.

Total GAG production (A) after 8 weeks, shown as GAGs retained in the construct or GAGs secreted into the media. The GAGs retained in the constructs were normalised to the DNA content (B) after 8 weeks culture. Bars and error bars show the means and standard deviations of 4 samples, respectively. Groups without a common Roman numeral are statistically different (p < 0.05). In panel A, lower and upper cases were used for GAGs secreted and retained, respectively.
**Fig. 4.** Micro-CT scans of cell-free hydrogel constructs (A, E) or cell laden constructs (B – D, F – H). Constructs were scanned after 2 weeks, (A, B, E, F), 5 weeks (C, G) or 8 weeks culture (D, H). Constructs were Gel-MA (A – D) or G-HA (E – H). The scale bar in G is 2 mm and the scale is the same for all panels. The attenuation scale in H ranges from 10,000 (blue) to 22,000 (red), and applies to all panels. Fig. S4-6 show the images for all constructs at the time points evaluated.
Fig. 5. Relative expression levels of collagen type II (A), aggrecan (B), collagen type I (C), PRG4 (D), collagen type 10 (E) and MMP13 (F) at day 0 and after 5 weeks culture. Expression of each gene was normalised to the Gel-MA control, and presented on a log₂ scale. Box plots show data from 4 samples, and groups without a common Roman numeral are statistically different.
Fig. 6. Compressive moduli (A, B) and wet weights (C, D) of cell-free hydrogels (A, C) and cell-laden hydrogel constructs (B, D). Compressive moduli and wet weights are shown after 1 day, 5 weeks and 8 weeks of culture. Bars and error bars show the means and standard deviations of 4 samples, respectively. At day 1 and week 8, statistically significant differences between the hydrogel groups are indicated by different Roman numerals, using lower and upper case, respectively. At week 8, # indicates a significant difference from day 1, while * indicates that the change during culture is significantly different to the change of corresponding gel-MA constructs.

Table 1
Composition and notation of hydrogels tested in this study.

<table>
<thead>
<tr>
<th>Composition (% w/v)</th>
<th>Notation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Gel-MA</td>
<td>Gel-MA</td>
</tr>
<tr>
<td>9.5% Gel-MA, 0.5% HA-MA</td>
<td>G-HA</td>
</tr>
<tr>
<td>9.5% Gel-MA, 0.5% CS-MA</td>
<td>G-CS</td>
</tr>
<tr>
<td>9% Gel-MA, 0.5% HA-MA, 0.5% CS-MA</td>
<td>G-HA-CS</td>
</tr>
</tbody>
</table>
Fig. S1. Morphologies of chondrocytes that were encapsulated (A) or at the surface (B) of a G-HA construct, and collagen type I immunofluorescence showing strong staining at the edge of a G-HA-CS construct (C). Constructs were cultured for 8 weeks, and in A and B, cell membranes were stained using cell mask (green) and nuclei were counterstained with DAPI (blue). Scale bars in A and B are 50 μm, and C is 100 μm.

![Image of morphologies](image1.png)

Fig. S2. Total GAG content at 8 weeks in cell-free hydrogels (A) and hydrogel constructs with middle/deep chondrocytes (B). Bars and error bars show the means and standard deviations of 4 samples.

![Image of GAG content](image2.png)

Fig. S3. Cumulative GAG release into culture media from cell-free gels (A), and corrected cumulative GAG release from chondrocyte laden constructs (B). Corrected values were calculated as the difference between the amount of GAGs secreted from cell-laden constructs and cell-free gels. Each data point represents the mean of 4 samples.

![Image of GAG release](image3.png)
**Fig. S4.** Change in swelling ratios of cell-laden hydrogel constructs during 8 weeks culture. Groups without a like Roman numeral are statistically different (p < 0.05).

**Fig. S5.** EPIC-μCT scans of hydrogel constructs cultured for 2 weeks. The scale bar represents 2 mm, and the same scale applies to all panels. The attenuation range is 5,000 – 22,000 in all panels.
Fig. S6. EPIC-μCT scans of hydrogel constructs cultured for 5 weeks. The scale bar represents 2 mm, and the same scale applies to all panels. The attenuation range is 5,000 – 22,000 in all panels.

Fig. S7. EPIC-μCT scans of hydrogel constructs cultured for 8 weeks. The scale bar represents 2 mm, and the same scale applies to all panels. The attenuation range is 5,000 – 22,000 in all panels.