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Covalent incorporation of heparin improves chondrogenesis in photocurable gelatin-methacryloyl hydrogels^a

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Multicomponent Gelatin-methacryloyl (GelMA) hydrogels are regularly adopted for cartilage tissue engineering (TE) applications, where optimising chemical modifications for preserving bio-functionality is often overlooked. In this study, we investigate the biological effect of two different modification methods, methacrylation and thiolation, to co-polymerise GelMA and heparin. The native bioactivity of methacrylated heparin (HepMA) and thiolated heparin (HepSH) is evaluated via thromboplastin time and heparan sulfate-deficient myeloid cell-line proliferation assay, demonstrating that thiolation is superior for preserving anticoagulation and growth factor signalling capacity. Furthermore, incorporating either HepMA or HepSH in chondrocyte-laden GelMA hydrogels, cultured for 5 weeks under chondrogenic conditions, promote cell viability and chondrocyte phenotype. However, only GelMA-HepSH hydrogels yield significantly greater differentiation and matrix deposition *in vitro* compared to GelMA. This study demonstrated that thiol-ene chemistry offers a favourable strategy for incorporating bioactives into gelatin hydrogels as compared to methacryloylation while furthermore highlighting GelMA-HepSH hydrogels as candidates for cartilage TE applications.

1. Introduction

At the interface of basic molecular biology research, chemistry, and biomaterial fabrication strategies, hydrogels provide a highly adaptable and cell cytocompatible environment that can be easily manipulated, typically through covalently incorporating various cell instructive elements to facilitate differentiation and tissue growth mechanisms targeting tissue engineering and regenerative medicine (TERM) strategies.^[1-7] This rationale has motivated a great number of researchers to develop unique biomimetic hydrogels in pursuit of a blueprint to bridge the gap between engineered- and native tissues.^[1-18] Recent advances within the TERM discipline have focused on the use of gelatin as a hydrogel platform given its versatility, inherent biological complexity as well as its suitability and rheological properties for use in biofabrication.^[10, 19-23] The favourable properties of gelatin and its rationale as a biomaterial hydrogel are well established, given that gelatin is a water-soluble protein containing cell adhesion domains, is enzymatically degradable and cheap to produce.^[19, 22-25] Furthermore, gelatin is easy to modify due to its abundance of inherent amine, hydroxyl and carboxyl groups, which can be grafted with methacryloyl groups (methacrylamides and methacrylates) thereby allowing radical initiated photopolymerisation for hydrogel fabrication.^[26, 27] However, gelatin-methacryloyl (GelMA) hydrogels alone have shown inadequate promotion of long-term chondrogenesis. For example, chondrocytes encapsulated in

pure GelMA demonstrate limited re-differentiation capability with deposition of predominantly a collagen type I ECM, which is indicative of fibrous tissue phenotype and poor tissue quality.^[9, 28] Recent studies have covalently incorporated chondrogenic compounds, such as hyaluronic acid (HA), chondroitin sulphate (CS) and decellularised ECM components (dECM), into GelMA hydrogels, as an approach to enhance the chondrogenic differentiation of encapsulated chondrocytes within these hydrogels.^[3, 9, 29] These studies show the potential of incorporating instructive elements in GelMA hydrogels for cartilage tissue engineering purposes while also highlighting the many unknown design criteria and parameters affecting tissue formation *in vitro*.

Firstly, the majority of the current biomimetic compounds investigated to support intricate cellular processes in combination with GelMA are proteoglycans found in mature cartilage (e.g. CS, HA, dECM) as opposed to developmental stage-specific ECM molecules (small leucine-rich repeat proteoglycans or heparan sulphate).^[30-35] Moreover, the present focus has been to include single biological cues rather than mimicking the native ECM's role to administrate growth factor activity by binding, storing and releasing them. In an effort to replicate the presentation and administration of biochemical cues by the ECM, one approach is to covalently incorporate heparin into hydrogels. Heparin is a glycosaminoglycan (GAG) and structural analogue to heparan sulfate, an important factor in cartilage development. Heparin has been previously shown to improve the re-differentiation capacity of chondrocytes,^[36] in addition to its capacity to bind various fibroblastic growth factors (FGFs)^[37] that may influence paracrine signaling. Despite the large body of work investigating bioactivity as well as cellular and tissue response of heparin/heparan sulphate, to our knowledge, heparin has been overlooked as a bioactive compound for incorporation within GelMA hydrogels.

Secondly, typical molecular incorporation approaches require chemically conjugating functional groups onto the targeted biologics to ensure their stable integration into GelMA hydrogels. However, the impact of these modifications on the native bioactivity of resultant hydrogels is not often considered. Methacryloylation is an extensively used modification protocol to covalently incorporate biochemical cues in GelMA hydrogels via radical initiated chain growth copolymerization. For instance, Levett *et.al.* previously reported that the successful incorporation of hyaluronic acid (HA) into GelMA hydrogels via UV photo-polymerisation improved both mechanical properties and chondrogenic cell morphology, however no significant difference in GAG formation was observed after both 4 and 8 weeks *in vitro* culture. Although photo-polymerisation of methacryloyl groups thus proves to be a reliable and widely used platform, this chemical modification may in fact cause cytotoxicity^[38] and structural damage to the modified matrix components and needs to be carefully assessed for each biological application. In parallel, Benoit *et.al.* discovered that grafting heparin with methacrylates resulted in a reduction of ability for heparin to bind to proteins in accordance with increasing degree of modification (DoM).^[39] The current chemical modification strategy commonly used to fabricate multi-component GelMA based hydrogels may thus be limiting the native bioactivity, questioning the validity of incorporating biological cues. Other hydrogels based on poly(ethylene glycol) (PEG) have instead been successfully crosslinked with thiolated molecules via mixed-mode polymerisations. Such an approach may offer a more compatible protocol for biological applications via the presentation of already natural occurring thiol moieties compared to methacryloyl groups.^[7, 40-44] Still, the potential of adopting thiol chemistry in regulating bioactivity, viscoelasticity and physicochemical properties of multi-component GelMA hydrogels designed for cartilage TE has not been orderly investigated.

In this study, we aimed to design a pro-chondrogenic environment by covalently incorporating heparin into GelMA hydrogels. Specifically, we adopted a systematic approach to compare the effect of thiolation and methacryloylation of heparin on native bioactivity, retention, and ability to promote chondrogenesis in photo-polymerised GelMA-HepMA and GelMA-HepSH hydrogels laden with human articular chondrocytes.

2. Experimental Section

2.1. Materials and reagents

Gelatin (porcine skin, type A, gel strength 300), heparin sodium salt (porcine intestinal mucosa, grade I-A), methacrylate anhydride (MAAh), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 1-hydroxybenzotriazole hydrate (HOBt), cysteamine, dialysis tubing cellulose membrane (MWCO 14 kDa), proteinase K, Trizma® base, ethylenediaminetetraacetic acid disodium salt dihydrate (Disodium-EDTA), 1,9-dimethyl-methylene blue zinc chloride double salt (DMMB), chondroitin-4-Sulfate, 4% neutral buffered formalin, glycine, sodium chloride (NaCl), phosphate buffered saline (PBS), sodium azide, hyaluronidase, ITS+1, L-proline, L-ascorbic acid-2-phosphate sesquimagnesium salt (AsAp), dexamethasone, fast green FCF, safranin-O, hydrochloric acid (37%), sodium hydroxide (NaOH), sodium bicarbonate, β-mercapto-ethanol and primary antibody for Connexion43 (C6219) were all purchased from Sigma-Aldrich, St Louis, MO. Trypsin-EDTA solution, CyQuant® cell proliferation assay kit, Gibco® DMEM hi-glucose glutaMAX™ media (10569-010), bovine serum albumin (BSA), AlamarBlue®, Gibco 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Gibco® non-essential amino acids (NEAA), penicillin-streptomycin (10,000 U/mL and 10,000 µg/mL), Gibco® fetal bovine serum (FBS), Gibco® RPMI 1640 medium, goat-anti-mouse secondary antibody

(Alexa Fluor 488), 4,6-diamidino-2-phenylindole (D1306, DAPI), molecular probes calcein-AM and propidium iodide, fibroblast growth factor 2 (FGF2), and BD Vacutainer 3.2% sodium citrate blood collection tubes was obtained from Thermo Fisher Scientific, Auckland, NZ. Primary antibodies collagen II (II-II6B3-C) were acquired from DSHB, Iowa City, USA. Primary antibodies for collagen I (Ab34710) and aggrecan (ab3773) was obtained from Abcam, Melbourne, Australia. Transforming growth factor β -1 (TGF β -1) was purchased from R&D systems, Minneapolis, USA. Type II collagenase was purchased from Worthington biochemical corporation, Lakewood, USA. Di-sodium hydrogen phosphate (Na₂HPO₄), acetic acid (glacial, 100%), and Gill's hematoxylin was purchased from Merck Millipore, North Shore City, NZ. MTS cell proliferation reagent was obtained from Promega, Madison, Wisconsin, USA, Tissue-Tek[®] O.C.T Compound Sakura[®] Fintek was purchased from VWR International, Radnor, USA, and 2-hydroxy-1-[4-(hydroxyethoxy)-phenyl]-2-methyl-1-propanone (12959) was a generous gift from BASF (David Wilkie, Manager Australia, and New Zealand). BaF3 cells, expressing the fibroblast growth factor receptor 2c (FGFR2c), were generously provided by Dr. David M. Ornitz of Washington University, St. Louis, MO. PMI 1640 media was purchased from MediaTech (Manassas, VA).

2.2. Macromer synthesis

Gelatin-methacryloyl (GelMA) and methacrylated heparin (HepMA) were synthesized using protocols based on methods described previously.^[26, 45] Briefly, gelatin and heparin were dissolved in PBS at 10 wt% and 1 wt%, respectively, and allowed to react with 0.6 g MAAh per gram of gelatin and with a 10-fold molar excess MAAh over hydroxyl groups found in heparin, correspondingly. The reaction of gelatin and MAAh was allowed to continue for 1 hour at 50 °C under constant stirring while reaction mixtures of heparin and MAAh were kept at 4 °C under continuous stirring and repeatedly adjusting the pH to 8 during 24 h. Thiolated heparin (HepSH) was synthesized using

protocols based on previously described methods.^[46] In short, heparin was dissolved in PBS at 1 wt% and allowed to react with EDC, HOBT, and cysteamine, with 3.5-, 2.5-, and 3-fold molar excess over carboxylic groups, respectively. The pH was adjusted to 6.8 and the reaction was allowed to continue for 5 h at RT. All reaction solutions were dialyzed (MWCO 14,000 Da) against deionized water in order to remove unreacted molecules. All functionalized polymers were sterile filtered and lyophilized followed by storage at -20 °C until use.

A

B

C

Auth

Figure 1. Synthesis of GelMA (A), HepMA (B), and HepSH (C).

2.3. Macromer structural characterization

The DoM, defined as the percentage of reactive groups that are modified, was determined using ^1H -NMR according to previously described protocols.^[47, 48] The composition of acid treated porcine skin gelatin was acquired from the gelatin handbook of the gelatin manufacturer's institute of America (GMIA). For GelMA, the $\delta = 7.40\text{-}7.18$ ppm peaks were assigned the aromatic amino acids in gelatin, 2.1% phenylalanine, and thus calibrated to 10.5 protons. Lysine (4.1%) and hydroxylysine (1%) both contain the reactive $\alpha\text{-NH}_2$ sites and the $\delta = 5.71\text{-}5.60$ and $5.47\text{-}5.36$ ppm peaks correspond to the newly formed methacrylate hydrogens that are used to calculate the DoM. For HepMA and HepSH, the DoM was quantified by normalizing the ^1H -NMR spectra to the $\delta = 2.03$ ppm peaks, assigned the N-acetyl protons. Subsequently, HepMA peaks $\delta = 6.2$ and $\delta = 5.7$ corresponds to the newly formed vinyl methylene and HepSH peaks $\delta = 1.2, 2.9$, and 3.1 corresponds to the newly thiol and methylene protons and are used to calculate the DoM based on the ratio of the thiol or methylene protons integral to the internal standard protons.

The thiol content of functionalized heparin was also confirmed using Ellman's assay (Equation 1) with cysteamine as a standard curve, according to previously described protocols.^[49] Briefly, known amounts of HepSH and cysteamine were dissolved in PBS and Ellman's reaction buffer was prepared by mixing 100 mM sodium phosphate with 1 mM di-sodium-EDTA at pH 8. Ellman's reagent was prepared by mixing 5,5'-Dithio-bis-(2-nitrobenzoic acid) at 4 mg/mL in Ellman's reaction buffer. 50 µl of each sample dilution were mixed with 500 µl reaction buffer and 10 µl Ellman's reagent, incubated at RT for 15 min before measuring the absorbance at 412 nm using a spectrophotometer (Varioskan, Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA).

$$\text{DoM}_{\text{Ellman's}}(\%) = 100(n_t/n_c) \quad (1)$$

Where n_t is the amount of free thiols and n_c the amount of carboxyl groups present in the samples.

2.4. Activated Partial Thromboplastin Time

Heparin is known for its capacity to bind to and activate antithrombin III (AT), which successively facilitates the inactivation of factor Xa and consequently the anticoagulation cascade. As such, the well-established heparin-AT complex was used a model system to quantify to what degree the two modification protocols – methacrylation or thiolation – may impede the native anticoagulant biofunctionality of heparin. An Activated Partial Thromboplastin Time (aPTT) assay was used to determine the anticoagulant properties of unmodified heparin (Hep), HepMA, and HepSH. Platelet poor plasma (PPP) was obtained by collection of blood samples in 3.2% sodium citrate tubes from a healthy volunteer with the following double centrifugation method. The collected blood tubes were centrifuged (Eppendorf 5810R) at 1500 g for 10 min at 4 °C after which 75% of the platelet rich

plasma supernatant was aspirated into a sterile falcon tube and centrifuged again at 1750g for 10 min at 4 °C to remove platelets and leave PPP. For a second time, 75% of the supernatant was aspirated using aseptic techniques and then stored at -20 °C for less than one week. Concentration gradients (10, 30, 50, 100, 150 and 250 µg/mL) of Hep, HepMA, and HepSH were prepared by dissolving the macromers in 0.9 wt% NaCl in deionized water and 40 µl to 360 µl thawed PPP was added and then incubated at 37 °C. Using an automated haemostasis testing system (ACL TOP 700 CTS), 0.45 mg/mL micronized silica and 15 µM/mL calcium was added for the onset of the intrinsic pathway of coagulation. The time from the addition of calcium to electronic optical detection of a fibrin clot was determined as the clotting time.

2.5. BaF32 cell proliferation and growth factor signaling assay

A BaF32 assay was used to quantify the capacity of heparin to form a ternary complex with the fibroblastic growth factor receptor (FGFR) 1c and FGF-2, an important factor for modulating both the onset of hypertrophy and the TGFβ signaling pathways.^[50-52] BaF32 cells, an IL-3 dependent and heparan sulphate deficient myeloid B cell line that has been stably transfected with FGFR1c,^[53, 54] were maintained in RPMI 1640 medium containing 10% (v/v) FBS, 10% (v/v) WEHI-3BD conditioned medium and 1% (v/v) penicillin-streptomycin. BaF32 cells thus need the addition of both heparin and FGF-2 to form a ternary complex with FGFR1c, resulting in cell proliferation. WEHI-3BD cells were maintained in RPMI 1640 medium supplemented with 2 g/L sodium bicarbonate, 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin and the conditioned medium was collected 3 times per week and stored at -20 °C until required. For the mitogenic assays, the BaF32 cells were transferred into IL-3 depleted medium for 24 h prior to experimentation and seeded into 96-well plates at a density of 2×10^4 cells/well in the presence of FGF-2 (0.3 nM) and 30 nM of either heparin, HepMA or HepSH. Cells were incubated for 48 h in 5% CO₂ and 95% air at 37 °C and the proliferation was determined

by an MTS colorimetric assay according to manufacturer's instructions, and the absorbance at 490 nm (Thermo Scientific Varioskan Flash).

2.6. Hydrogel fabrication

All hydrogel precursor solutions were prepared using PBS, supplemented with a final photo-initiator concentration of 0.05 wt% Irgacure® 2959 (I2959), injected into a custom-built Teflon mould that was covered by a glass slide to minimize the effects of oxygen inhibition,^[55] and irradiated with 365 nm ultraviolet light at an intensity of 3.6 mW/cm² for 15 min (Thermo Fisher Varioskan Flash). All fabricated hydrogels had initial dimensions of approximately 6 mm in diameter and 2 mm in height.

2.7. Quantification of macromer retention in cell-free hydrogels

Four hydrogel compositions, as shown in Table 1, were polymerized and incubated in PBS, containing 0.1 wt.% sodium azide, at 37 °C for up to 14 days. At each time point, hydrogel samples were removed from their PBS liquid and digested at 56 °C in 1 mg/mL proteinase K, dissolved in 10 mM Tris-HCl and 1mM disodium EDTA solution. The PBS incubation liquid and its matching digested hydrogel sample were both allowed to react with DMMB to quantify the heparin content. In brief, 50 µl of each sample and standard curve dilutions were transferred in triplicates to a 96-well microplate, to which 200 µl of DMMB solution was added and the absorbance was read twice at 520 nm (Thermo Scientific Varioskan Flash). Heparin content in both hydrogels and surrounding PBS liquid could be calculated from a matching standard curve generated by reacting known amounts of Heparin, HepMA, and HepSH with the DMMB agent. The retention, defined as the percentage of macromer not released to the surrounding liquid, could then be calculated for each time point using the following equation:

$$\text{Macromer retention (\%)} = 100[m_g/(m_g + m_l)] \quad (2)$$

Where m_g is the mass of heparin macromers found in the digested hydrogels and m_l is the mass of heparin macromers found leached out to the surrounding PBS that the corresponding hydrogel was submerged in.

Table 1. Hydrogel compositions and nomenclature.

Nomenclature	GelMA [Wt%]	Heparin [Wt%]
GelMA	10	-
GelMA-HepSH	9.5	0.5
GelMA-HepMA	9.5	0.5
GelMA-Heparin	9.5	0.5

2.8. Characterization of sol fraction

After polymerisation, each hydrogel was weighed (m_{iw}) and three samples per hydrogel composition were directly lyophilized to record their initial dry weights (m_{id}) and determine the actual macromer weight fraction ($m_{\%}$), which is reported as the ratio of the initial dry weight to the initial weight.

$$m_{\%} = m_{id}/m_{iw} \quad (3)$$

To determine the initial dry weight of the remaining samples, the factor of the actual macromer fraction and individual initial weight was used.

$$m_{id} = m_{\%}m_{iw} \quad (4)$$

The remaining samples were allowed to swell in PBS solution, containing 0.1 wt% sodium azide, at 37 °C for 1 day. Swollen hydrogel samples were collected to record wet weight (m_s), then lyophilized to obtain the freeze-dried weight (m_d) and the mass loss and mass swelling ratio (q) was calculated according to the following calculations:

$$\text{Mass loss} = 100(m_{id} - m_d)/m_{id} \quad (5)$$

$$q = m_s/m_d \quad (6)$$

The sol fraction of the hydrogels is defined as macromer not crosslinked into the hydrogel network and determined as the mass loss after equilibrium swelling ($t=1d$).

2.9. Cell isolation, expansion, encapsulation and culture

Chondrocytes were harvested from macroscopically normal regions of articular cartilage with consent from human patients undergoing ACL reconstruction (Health and Disability Ethics Committee New Zealand, URB/07/04/014/AM02). The tissue was digested in chondrogenic expansion media (Gibco® DMEM high glucose glutaMAX™ supplemented with 0.4 mM L-proline, 10 mM HEPES, 0.1 mM NEAA, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.1 mM AsAp and 10% FBS) with 0.15% type II collagenase at 37 °C in a humidified air incubator (5% CO₂/95% air) for 16-20 h. The solution was filtered through a cell strainer and isolated chondrocytes were expanded (525.000 cells/cm²) into high-density monolayers for two passages cultured in chondrogenic expansion media. Cells were then encapsulated in hydrogels at a concentration of 15 x 10⁶ cells/mL and cultured for 5 weeks in chondrogenic differentiation media (Gibco® DMEM high glucose glutaMAX™ supplemented with 0.4 mM L-proline, 10 mM HEPES, 0.1 mM NEAA, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.2 mM AsAp, 1 x ITS+1 premix, 1.25 mg/mL BSA, 10 nM dexamethasone and 10 ng/mL TGFβ-1). Cell free hydrogels served as a negative control group.

2.10. Viability assay

Live/dead assay was performed at two time points to visualize cell viability. In brief, hydrogel samples were washed with PBS, cut in half with a scalpel, and allowed to incubate with 1 µg/mL Propidium Iodide (PI) and 1 µg/mL Calcein-AM (Cal-AM) in PBS for 20 min at RT. After staining, samples were washed with PBS and left hydrated for imaging using a Zeiss Axioimager Z1 fluorescent microscope (Carl Zeiss Microscopy, Jena, Germany). The digital images were further processed using built in functions in ImageJ (version 6.1 Fiji, National Institutes of Health) to minimize the risk of tiny specs of signal, representing cell debris, hydrogel background noise or instrument artifacts, wrongfully being included in the cell counting. *Process – subtract background* was first applied to correct for autofluorescence background. *Image – adjust – threshold* was then used to ensure that enough area of signal was present to identify cells and their nucleuses. Finally, the *Analyse – analyse particles* function was employed to quantify the number of cells in the processed image. A constant pixel background subtraction, threshold level and area exclusion filter was applied for each of the three steps, respectively. To minimize stochastic errors, an iterative process verifying the automatic counting with manual counting ensured the optimisation of these three constants. Live (n_{live}) and dead (n_{dead}) cells were counted at three locations within the centre of each construct per time point. Viability was calculated accordingly.

$$\text{Cell viability (\%)} = 100[n_{\text{live}}/(n_{\text{live}} + n_{\text{dead}})] \quad (7)$$

2.11. Metabolic activity

AlamarBlue® was used to track the health of embedded chondrocytes once a week. Briefly, each hydrogel sample was incubated with 330 µl AlamarBlue solution for 4 h. The reduction of AlamarBlue® in the solution was determined by reading absorbance at wavelengths of 570 nm and 600 nm using a spectrophotometer (Thermo Scientific Varioskan Flash) followed by data processing according to manufacturer's instructions.

2.12. Biochemical analysis

Cell-laden hydrogel constructs were collected after 1 day and 5 weeks of culture in chondrogenic differentiation media. Samples were washed in PBS, weighed, lyophilised, weighed again prior to digestion at 56 °C in 1 mg/mL proteinase K, dissolved in 10 mM Tris-HCl and 1 mM disodium EDTA solution. The GAG content was quantified by allowing samples to react with the DMMB solution, as previously described,^[55, 56] followed by measuring the absorbance at 520 nm (Thermo Scientific Varioskan Flash). GAG content was calculated from a matching standard curve generated by reacting known amounts of chondroitin sulfate and subtracting the values obtained from cell-free controls. Total DNA was quantified using CyQUANT® Cell Proliferation Assay Kit according to manufacturer's instructions and as described previously,^[55, 56] following a pre-treatment of each sample with DNase-free RNase A (Thermo Fisher) to eliminate the RNA components of the fluorescent signal.

2.13. Histology

Cell-laden hydrogel constructs were collected after 1 day and 5 weeks, washed with PBS, followed by fixation in 4% formaldehyde for 1 h at RT and washed in PBS. The samples were then embedded in optimal cutting temperature compound (O.C.T) and cryo-sectioned into 30 µm thick sections. Haematoxylin, fast green, and Safranin-O was applied to visualise cell nuclei and extracellular GAGs using a Zeiss Axioimager Z1 microscope.

2.14. Histology and Immunofluorescence examination

Cell-laden hydrogel constructs were collected after 1 day and 5 weeks, washed with PBS, followed by fixation in 4% formaldehyde for 1 h at RT and washed in 0.1 M glycine in PBS to quench free aldehyde groups. Samples were incubated in 0.1% hyaluronidase for 30 min at RT and washed with PBS to reveal antigen epitopes. Constructs were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at RT. Primary antibodies for collagen I, collagen II, aggrecan, or connexin43 were diluted (1:200) in blocking buffer and applied overnight at 4 °C. Samples were washed three times in blocking buffer for 10 min each followed by incubation with a goat-anti-mouse (Alexa Fluor® 488) and donkey-anti-rabbit (Alexa Fluor® 594) secondary antibodies, diluted in blocking buffer (1:400), in the dark for 1 h at RT. Constructs were washed a further 3 times with blocking buffer before incubation with blocking buffer containing 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, 1:1000 dilution) in the dark for 1 h at RT. Lastly, constructs were washed three times in PBS and stored hydrated at 4 °C overnight. Images were captured using an inverted Leica TCS SP5 confocal microscope (Leica camera AG, Wetzlar, Germany).

2.15. Statistical analyses

All samples (n=9) were prepared in triplicates and each experiment was repeated three times, unless otherwise stated, to study variability between batches. Differences between heparin and hydrogel groups were assessed by one-way ANOVA. Independent sample t-test was used to compare data within groups or between time points, assuming unequal variances. All values are reported as the mean and standard error of the mean. Differences between data were considered significant when $p < 0.05$ for all tests.

3. Results and Discussion

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3.1. Macromer structure and biological activity

Primary amines and hydroxyl groups on the gelatin chain were successfully grafted with methacryloyl groups (**Figure S1**). Similarly, hydroxyl and carboxyl groups on heparin chains were successfully substituted with methacryloyl or thiol pendent groups. The DoM, specified as the amount of repeating units that have been modified, was determined to be $60 \pm 2\%$, $59 \pm 2\%$ and $61 \pm 7\%$ for GelMA, HepMA and HepSH, respectively, using $^1\text{H-NMR}$ and Ellman's assay. As such, relative to the current ranges for GelMA reported in literature, this study applies a medium range of DoM.^[19, 22, 26, 39, 45, 46, 48, 57]

An aPTT assay was performed to examine if the conjugation of these functional groups had compromised the capacity of heparin macromers to bind to, and activate, the AT complex in human plasma compared with non-modified heparin. Heparin and the two modified heparin (HepMA or HepSH) samples were added at various concentrations and the time to fibrin clot formation was determined. If the time exceeded 120 seconds, the results were ruled as complete inhibition of thrombin via activation of AT. Without heparin, clot formation was observed after 30 seconds, which is well within the normal reference range of the assay (58). As expected, it was observed that increased heparin concentration generated prolonged clotting times. Results demonstrated that greater than $3 \mu\text{g/mL}$ of non-modified heparin was sufficient to fully inhibit clot formation, while greater than $5 \mu\text{g/mL}$ of HepSH and $10 \mu\text{g/mL}$ of HepMA was required to generate the same inhibitory effect on coagulation (**Figure 2**). These results suggest that the specific AT binding site was affected by the chemical modifications, with methacryloylation significantly more so.

Another heparin binding molecule is FGF-2, which is known to impact hypertrophy, differentiation and guide stem cells down a chondrogenic lineage by postulated interplay with $\text{TGF-}\beta$ and early activation of the chondroinductive program.^[50, 51, 59] The ability of functionalized heparin, via either

methacryloyl or thiol groups, to retain its FGF2 signalling capacity was evaluated using a BaF-32 growth factor signalling assay. BAF-32 cells cultured in the presence of both heparin and FGF2 were used as positive controls, while cells in the presence of medium, FGF2 or heparin alone were used as negative controls. As the BAF-32 cells are a heparan sulphate deficient cell line, they lack the ability to form a ternary FGF-FGFR-heparin complex at the cell surface. As such, the cells are unable to proliferate on their own and, hence, are an attractive model system to identify heparan sulphate/heparin structures that interact with FGFs and their receptors forming the required ternary complex for FGF-2 mediated signalling. Results demonstrated that, relative to non-modified heparin, thiol modification of heparin did not affect the formation of the ternary FGF-FGFR-heparin complex at the cell surface ($p \geq 0.62$) to the same degree as methacryloylation ($p \leq 0.04$), as seen in **Figure 3**. Taken together, we hypothesise that since methacryloyl groups differ in both charge and hydrophobicity, compared to the naturally occurring and hydrophilic thiols, methacryloylation may have elicited hierarchical changes or perhaps obscured the active binding sites of interest.

Figure 2. Anticoagulation properties of Hep, HepMA, and HepSH measured with an aPTT assay. A long clotting time reflects that the heparin macromer efficiently binds to and activates the AT complex in human plasma. A clotting time longer than 120 seconds is considered a comprehensive activation of AT resulting in complete inhibition of the coagulation cascade.

Figure 3. BaF32 cell proliferation with and without 0.3 nM FGF-2 and 30 nM heparin after 48 h of incubation. * indicates significant difference (p < 0.05).

3.2. Retention profiles

GelMA hydrogels were successfully fabricated with addition of either heparin, HepMA or HepSH, where a final retention of 0.5 wt.-% was targeted in accordance with current literature exemplar.^[9] A quantitative sulphated GAG assay was used to determine the retention of non-modified heparin, HepMA, and HepSH in cell-free GelMA hydrogels. Results demonstrated that the retention of non-modified heparin was low ($8 \pm 1\%$), indicating a quick release from the hydrogel, while covalent incorporation of both HepMA and HepSH was successful (**Figure 4A**). Retention data for HepMA in this study ($85 \pm 3\%$) correlated well with current reports investigating methacrylated compounds incorporated in GelMA (e.g. HA and CS).^[9, 29] However, a significantly lower HepSH retention ($28 \pm 6\%$) in GelMA compared to HepMA was observed (Figure 4A). As a result, it is important to note that an adjustment of initial macromer fractions (0.67 wt% HepMA and 3.29 wt% HepSH) was subsequently undertaken to match the design criteria of 0.5 wt-% final heparin macromer fraction, and that these modified initial macromer fractions were then used for all subsequent GelMA-HepSH hydrogels throughout the remainder of this study.

One possible explanation for the observed differences in retention between HepMA and HepSH may relate to step growth thiol conjugation rivalling the chain growth polymerisation of methacryloyl groups, and that the coupling reaction of thiols appears very selective, likely due to slow or limited propagation kinetics. Firstly, the intermediate carbon-centered radical – formed following thiol radical addition to a methacryloyl moiety for example – is known to be relatively stable and could therefore limit the rate for hydrogen abstraction from the next thiol in the second stage of the thiol-ene propagation.^[60, 61] Secondly, the thiol-ene addition reaction only converts one methacryloyl and one thiol group to a single thiol-ether bond, while the methacryloyl co-polymerisation involves chain propagation along multiple methacryloyl groups. This chain polymerisation likely results in a faster

consumption of methacryloyl groups than what the two-step thiol-ene addition reaction can compete with.^[62] Thirdly, the conventional strategy used to apply cysteamine to conjugated polysaccharides with pendant thiol groups may lead to the formation of disulfide or polysulfide crosslinks, possibly initiating a thiol-disulfide exchange mechanism.^[63] This phenomenon would reduce the amounts of free thiols present and thus impede the incorporation of HepSH, yielding a low retention. This illustrates the need to optimize suitable photoinitiators and light sources for each thiol-ene system to achieve efficient thiol incorporation, and is a focus of our further investigations.

3.3. Sol fraction and mass swelling

The addition of heparin in GelMA hydrogels holds the potential to affect the physical microenvironment, possibly by acting as an antioxidant that quenches radicals and thus impairs the sol fraction. The addition of heparin may further introduce an increase in negative charges, affecting the swelling capacity of the crosslinked hydrogels.^[64, 65] It has been suggested that soft versus stiff hydrogels, as a result of sol fraction and mass swelling characteristics, plays a role in controlling extra cellular matrix secretion.^[66, 67] Therefore, it is important to characterise the hydrogel physico-chemical environment in order to distinguish between the impact of heparin on chondrogenesis as a result of alterations in hydrogel bioactivity, as compared to the physical attributes of the matrix. In the current study, cell-free hydrogels were weighed, lyophilized and weighed again directly after fabrication to calculate the actual macromer fraction (Equation 2). In parallel, remaining samples were allowed to soak in PBS at 37 °C for 24 h to characterise the sol fraction and swelling ratio. The crosslinking efficiency was comparable between all hydrogel compositions (**Figure 4B-C**), providing similar physico-chemical microenvironment initially with no significant difference observed for either sol fraction ($\approx 13\text{-}14\%$, $p > 0.98$) nor mass swelling ratio ($\approx 11\text{-}12\%$, $p > 0.36$) respectively. In contrary, the addition of heparin does affect the mechanical properties of the hydrogel constructs

(Figure 4D), where GelMA displays a significantly higher compressive modulus (29 ± 3 kPa, $p \leq 0.001$) compared to both GelMA-HepMA (21 ± 2 kPa) and GelMA-HepSH (22 ± 1 kPa), while no noteworthy difference was detected between incorporating HepMA compared to HepSH in GelMA hydrogels ($p \geq 0.480$). These results correlate well with literature values^[19, 55, 68] and further underlines that significantly increasing the amount of HepSH starting material (as required to achieve the targeted 0.5 wt% HepSH incorporation) does not result in a change in the overall sol fraction, initial swelling, or mechanical properties of the polymer network respectively. Adjustment of the initial macromer weight simply alters the absolute amount of HepSH covalently incorporated in GelMA hydrogels, matching the amount of HepMA covalently incorporated in GelMA. Nonetheless, covalently incorporating either of the modified heparins in the GelMA polymer network leads to the introduction of negative charges that could possibly result in fewer physical crosslinks or an increased capacity to retain water, explaining the significant decrease in compressive modulus observed. Li *et.al.* established that GelMA hydrogels with a compressive stiffness of approximately 30 kPa was favourable over both 17 kPa and 4 kPa hydrogels in terms of maintaining chondrogenic phenotype.^[69] As such, it would suggest that the slightly stiffer GelMA hydrogels (29 kPa), compared to both GelMA-HepMA (21 kPa) and GelMA-HepSH (22 kPa), observed in this study may offer a more favourable environment for modulation of chondrocyte phenotype via physical cues.

Figure 4. Retention of non-modified heparin, HepMA, and HepSH in GelMA hydrogels as a function of time (A). Heparin content was determined using a quantitative DMMB assay. Error bars represent the mean $\pm$ SD of 17 samples (n = 5 batches). Sol fraction (B), mass swelling ratio (C) and compressive modulus (D) of fabricated hydrogels after equilibrium swelling. * indicates significant difference ($p < 0.05$).

3.4. Cell viability and metabolic activity

One of several important characteristics of a biomaterial is the capacity to support cell viability and metabolic activity. Factors such as physical properties, chemical reactivity and toxicity of radicals

generated during the chemical crosslinking process, are all well-established factors impacting cellular health. Human articular chondrocytes were therefore encapsulated in GelMA, GelMA-HepMA and GelMA-HepSH hydrogels and cultured for 5 weeks to evaluate long-term cell viability and function. Cell survival was evaluated using a live/dead assay, where living cells are stained green while dead cells are stained red. Metabolic activity of chondrocytes encapsulated in the hydrogels was quantified using an AlamarBlue® assay. Results demonstrated that there was no significant difference observed in cell viability (1 day $\approx$ 81-86%, 5 weeks $\approx$ 74-78%, $p \geq 0.75$) between the conditions at any of the two time points (**Figure 5A-G**). Cell survival was high after one day of culture, primarily reflecting that the cells were able to survive the encapsulation process in all hydrogel conditions. Conversely, a significant decline in viability was noticed for both GelMA (from 86% to 76%, $p \leq 0.02$) and GelMA-HepSH (from 84% to 74%, $p \leq 0.01$) hydrogels after 5 weeks of culture. However, this was not mirrored in the metabolic activity profiles (**Figure 5H**), where instead a stagnation or overall increase with time was observed (1 day $\approx$ 67-77%, 5 weeks $\approx$ 79-87%). Furthermore, all hydrogel conditions exhibited a similar range of cell viability (74-86%) post-encapsulation and throughout the culture period, and correlates with previously reported viability for GelMA hydrogels using the I2959 UV-crosslinking platform.^[19, 22] As such, these satisfactory cell viability and metabolic activity maintenance observations during long-term *in vitro* culture indicate that all hydrogel mesh sizes and crosslinking densities generated in this study allow for appropriate solute diffusion.

Figure 5. Live/Dead images of GelMA (A, D), GelMA-HepMA (B, E) and GelMA-HepSH (C, F) hydrogel construct seeded with 15×10^6 cells/mL after 1 day (A-C) and 5 weeks (D-F). Green and red fluorescence indicated live and dead HACs, respectively. Scale bar = 100 μ m. Viability (G) and metabolic activity (H) as a function of time. * indicates significant difference (p < 0.05).

3.5. Cartilage matrix and DNA accumulation

One of the key characteristics of functional ECM and cartilage tissue formation is the synthesis and deposition of sGAGs and collagen II replicating levels in normal healthy articular cartilage.^[70, 71] For this purpose, the DMMB spectrophotometric assay was used to quantify glycosaminoglycan excretion while histology and immunofluorescent techniques were applied to visualize the extracellular matrix formation of encapsulated chondrocytes cultured for 5 weeks in chondrogenic differentiation media *in vitro*. Furthermore, DNA was quantified using the CyQuant® assay to indicate cell proliferation. DNA data was also used to normalize the GAG production to obtain a quantitative measure of chondrocyte differentiation capacity (GAG/DNA). DNA data indicate that both GelMA and GelMA-HepSH hydrogels support cell proliferation with a significant increase in DNA content over time (**Figure 6A**). This result correlates well with current literature findings, displaying an increase in DNA in cell-laden GelMA hydrogels within a 28-35 days culture period.^[3, 10, 29] In contrast, GelMA-HepMA hydrogels showed a reduction in cell number over 5 weeks in culture, indicating that the addition of HepMA alters the metabolism of the cells, and thus possibly restricts its use for cell-based cartilage repair strategies. This was further underlined by the large variations in GAG production observed in GelMA-HepMA hydrogels (**Figure 6B**). However, this could be due to the observed baseline levels of HepMA retained in the cell free hydrogels, as seen in **Figure 7**. It is challenging, both quantitatively and histochemically, to determine if GAG formation detected is the ECM produced by the encapsulated cells or if it is the covalently-incorporated HepMA background in the GelMA-HepMA cell-laden hydrogels. As GelMA is known to be enzymatically degradable, it could be argued however that the cell-free hydrogels, used as controls to quantify the baseline amount of heparin background detected by the DMMB assay in the cell-laden hydrogels, could potentially contain more heparin compared to the cell-laden constructs as they are not being degraded over time and thus retain all covalently incorporated heparin. Chondrocytes further hold the capability to

secrete mediators, such as nitric oxide which can be converted to nitrous acid, a compound known to degrade heparin and heparin sulphate.^[72-75] Nonetheless, applying cell free hydrogels at similar time points as a baseline to quantify cell secreted GAGs is the most appropriate strategy, as per current protocols.^[9] The large standard deviation for the GelMA-HepMA condition could also be due to remodelling of the ECM. However, immunohistological results show that the secretion of both collagen type I and collagen type II was limited in GelMA-HepMA hydrogels compared to the other two conditions (**Figure 8**). This, once again, correlates back to the stagnation in DNA observed over the 5 week culture time (Figure 6A). Although the cells present in the GelMA-HepMA hydrogels show signs of high matrix production per cell with both favourable collagen II/I ratios and GAG content, there was ultimately not enough ECM production to achieve high-quality cartilage tissue. Instead, DMMB results clearly show significantly greater GAG content in GelMA-HepSH constructs as compared to GelMA constructs (**Figure 6C**). This was supported by safranin-O stained cryosections visualizing the extent of the sGAGs accumulation (Figure 7). The cell-free GelMA-HepSH hydrogels do not stain positively for Safranin-O as very little HepSH is retained after 5 weeks, indicating the significant GAG secretion as being responsible by encapsulated cells in GelMA-HepSH hydrogels. Furthermore, substantial deposition of both collagen I and II could be detected in GelMA hydrogels while GelMA-HepSH hydrogels visually displayed larger depositions of collagen II (Figure 8) compared to collagen type I, an established trademark for hyaline cartilage. It is considered that these cell response modulations are dominantly affected by the biochemical cues present in the hydrogel network rather than the hydrogel stiffness recorded in this study. Combined, these results indicate that GelMA-HepSH was the only hydrogel condition that consistently promoted cell viability, proliferation, chondrogenic morphology, large deposition of GAG, as well as favourable collagen type II/I ratios throughout this study.

In future studies, it would be necessary to systematically and quantitatively study the underlying mechanism by which HepSH alters cell phenotype and matrix deposition. The effect of heparin on cellular differentiation is often correlated to the capacity to sequester, deliver and potentiate TGF- β ^[76,77] while the cocktail effect between FGF-2 and TGF- β is typically overlooked. It has herein been suggested that FGF signaling inactivates TGF- β signaling.^[78,79] It thus remains important to establish if HepSH induced chondrogenesis in GelMA hydrogels is attributed to HepSH binding, storing and releasing TGF- β or its ability to instead bind FGF-2, subsequently hindering FGF from inhibiting TGF- β accelerated chondrogenesis, combined or individually. Another key investigation lies in improving the HepSH retention in gelatin based hydrogels, possibly by studying alternative crosslinking and chemistry protocols. This is further necessary to compare the bioactivity of covalently incorporated HepSH to soluble additions as well as soluble and heparin bound growth factors such as FGF and TGF- β .

Figure 6. Quantification of DNA, GAG and GAG normalized to DNA content in GelMA, GelMA-HepMA and GelMA-HepSH cell laden hydrogels cultured in chondrogenic differentiation media. * indicates significant difference ($p < 0.05$).

Figure 7. Safranin-O stained cryosections of cell laden (left) and cell free (right) hydrogels collected after 5 weeks culture in chondrogenic differentiation media. Scale bar = 100 μ m.

Figure 8. Confocal immunofluorescent analysis of encapsulated cells in GelMA, GelMA-HepMA and GelMA-HepSH hydrogels after 5 weeks of culture in chondrogenic differentiation media using DAPI (blue), collagen type II (green) and collagen type I (red) antibodies. Scale bar = 100 μ m.

3.6. Paracrine signalling

The relationship between the addition of heparin, and subsequent FGF-2 growth factor signaling capacity in GelMA hydrogels, with gap junction protein connexin43 (Cx43) accumulation was further explored. It has been suggested in literature that chondrocytes exposed to TGF- β 1 produce noteworthy amounts of the FGF-2 growth factor.^[80] Previous studies have further shown that the expression of Cx43 and FGF-2 is highly interrelated, where it has been demonstrated that disruption of Cx43 expression limits cells responsiveness to FGF-2,^[81] while others have observed that FGF-2 exposure causes a reduction of Cx43-protein.^[82] The immunohistochemical evaluation in this study demonstrated that cells encapsulated in both GelMA and GelMA-HepSH hydrogels supported paracrine signaling (**Figure 9**). These results suggest that the addition of heparin does not clearly modulate gap junction formation. Further work is thus necessary to quantitatively establish if facilitating FGF-2 growth factor binding via the addition of heparin influences paracrine signaling, and subsequently the tissue formation in GelMA hydrogels.

Figure 9. Confocal immunofluorescent analysis of encapsulated cells in GelMA, GelMA-HepMA and GelMA-HepSH hydrogels using AggreCAN (green), Connexin-43 Antibody (red) and DAPI (blue). Scalebar = 100μm.

4. Conclusions

Gelatin-methacryloyl (GelMA) hydrogels are commonly adopted for tissue engineering applications (e.g. cartilage), and offer a versatile and cytocompatible hydrogel platform. However, to our knowledge, optimisation of chemical modification strategies for preserving biological function when incorporating bioactives in GelMA hydrogels have not been previously investigated. In this study, we systematically compared two different modification methods (methacryloylation and thiolation) to covalently incorporate heparin into GelMA hydrogels.

Heparin was successfully grafted with thiol or methacryloyl functional groups as per previously published syntheses protocols. We demonstrated that thiol-modification of heparin was a superior protocol for preserving the native anticoagulation and growth factor signaling capacities of heparin compared to methacryloylation. GelMA was successfully copolymerised with either HepMA or HepSH, with the latter revealing lower heparin retention when incorporated in the hydrogel. It was observed that the addition of small amounts of heparin in GelMA hydrogels offered the potential to facilitate cell viability and promote chondrocyte phenotype in encapsulated human articular chondrocytes. It also revealed that the reduced bioactivity of HepMA resulted in reduced proliferation, ECM accumulation, and ultimately uneven tissue formation following 5 weeks *in vitro* culture. In contrast, GelMA-HepSH hydrogels yielded significantly greater differentiation and matrix deposition compared to GelMA hydrogels. The findings also suggest that GelMA-HepSH hydrogels promoted collagen type II accumulation and connexin43 mediated gap junction communication.

This study demonstrated that combining GelMA and heparin is a promising hydrogel formulation for cartilage tissue engineering applications, further establishing heparin as a suitable cell instructive molecule and matrix component for chondrocyte-laden hydrogels *in vitro*. Additionally, results revealed that commonly adopted chemical modification strategies used currently to fabricate multi-component GelMA hydrogels may limit the native bioactivity of heparin. These findings highlight that achieving proper cellular responses in biomimetic gelatin hydrogels does not only rely on the selection of cell type, culture conditions and matrix components, but also the choice of functionalisation chemistries. Future studies should consider adapting thiol-ene chemistry for fabricating cell-instructive biomaterials and bioinks with retained bioactivity in order to investigate their potential for improved functional tissue formation.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Covalent incorporation of heparin into cell-laden gelatin methacryloyl hydrogels significantly improves cartilage tissue formation. We observe thiolation to be a more attractive chemical modification strategy that preserves heparin bioactivity compared to the almost ubiquitous application of methacrylation co-polymerisation protocols.

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Supporting Information

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Covalent incorporation of heparin improves chondrogenesis in photocurable gelatin-methacryloyl hydrogels

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Figure S1. ^1H -NMR spectra of gelatin (A), GelMA (B), heparin (C), HepMA (D), and HepSH (E) in D_2O .

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Figure S2. Cytoskeletal arrangement of chondrocytes encapsulated in GelMA, GelMA-HepMA, and GelMA-HepSH hydrogels at 5 weeks. Cell nuclei are shown in blue, membrane protein CD44 in Green and f-actin in red. **Scale bar = 100 μ m.**

Figure S3. Full section images of safranin-O stained cryosections of cell laden GelMA (A), GelMA-HepMA (B) and GelMA-HepSH (C) hydrogels collected after 5 weeks culture in chondrogenic differentiation media. Scale bar = 500 μ m.

Figure S4. Total GAG content normalized to dry weight in cell free and cell laden hydrogels (without baseline corrections) after 5 weeks of incubation in chondrogenic differentiation media.

5. Experimental section

5.1. Materials and reagents

Primary antibodies for CD44 (H4C4-C) were acquired from DSHB, Iowa City, USA. F-actin phalloidin-fluorescein (rhodamine phalloidin 594), and 4,6-diamidino-2-phenylindole (D1306, DAPI) was

obtained from Thermo Fisher Scientific, Auckland, NZ. 4% neutral buffered formalin and deuterium oxide was purchased from Sigma-Aldrich, St Louis, MO.

5.2. NMR

Modified and non-modified gelatin and heparin samples were dissolved in deuterium oxide and ^1H -NMR spectra were collected on a 500 MHz Varian UNITY INOVA spectrometer (Varian Inc., Palo Alto, CA, USA). The composition of acid treated porcine skin gelatin was acquired from the gelatin handbook of the gelatin manufacturer's institute of America (GMIA). ^1H -NMR spectra of gelatin (**Figure S1A**), GelMA (**Figure S1B**), heparin (**Figure S1C**), HepMA (**Figure S1D**), and HepSH (**Figure S1E**) in D_2O confirmed the success of MA and SH grafting. $\delta_f = 7.40\text{-}7.18$ (d, arom. $-\text{CH}$), $\delta_g = 5.71\text{-}5.60$ (m, $-\text{NH-CO-C}(\text{CH}_3)=\text{CH}_2$), $\delta_h = 5.47\text{-}5.36$ (m, $-\text{NH-CO-C}(\text{CH}_3)=\text{CH}_2$), $\delta_i = 2.15\text{-}1.85$ (m, $-\text{NH-CH}_2\text{-CO-CH}_3$), $\delta_j = 3.13\text{-}3.05$ (m, $-\text{CH}_2\text{-CH}_2\text{-SH}$), $\delta_k = 2.90\text{-}2.85$ (m, $-\text{CH}_2\text{-CH}_2\text{-SH}$), $\delta_l = 1.20\text{-}1.05$ (d, $-\text{CH}_2\text{-CH}_2\text{-SH}$), $\delta_m = 6.55\text{-}6.25$ (m, $-\text{O-CO-C}(\text{CH}_3)=\text{CH}_2$), $\delta_n = 5.7\text{-}5.6$ (m, $-\text{O-CO-C}(\text{CH}_3)=\text{CH}_2$). Spectra B confirm the existence of methacryloyl groups in GelMA, since typical signals at δ_b and δ_c are present, which were not found in the spectrum of original gelatin (**Figure S1A-B**). A slight shifting of some resonance peaks also occurred. Spectra D confirm the existence of methacryloyl groups in HepMA, since typical signals at δ_h and δ_i are present, and thiol groups in HepSH, since typical signals at δ_e , δ_f , and δ_g are present, which were not found in the spectrum of original heparin (**Figure S1C**).

5.3. Immunohistochemistry

Cell-laden hydrogel constructs were collected after 1 day and 5 weeks, washed with PBS, followed by fixation in 4% formaldehyde for 1 h at RT and washed in 0.1 M glycine in PBS to quench free aldehyde groups. Samples were incubated in 0.1% hyaluronidase for 30 min at RT and washed with PBS to reveal antigen epitopes. Constructs were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at RT. Primary antibodies for CD44 were diluted (1:200) in blocking buffer and applied

overnight at 4 °C. Samples were washed three times in blocking buffer for 10 min each followed by incubation with a goat-anti-mouse (Alexa Fluor® 488) secondary antibodies, diluted in blocking buffer (1:400), in the dark for 1 h at RT. Constructs were washed a further 3 times with blocking buffer before incubation with blocking buffer containing F-actin Phalloidin-fluorescein (1:500 dilution) and DAPI (1:1000 dilution) in the dark for 1 h at RT. Lastly, constructs were washed three times in PBS and stored hydrated at 4 °C overnight. Images were captured using an inverted Leica TCS SP5 confocal microscope (Leica camera AG, Wetzlar, Germany). Various cytoskeletal arrangements could be observed in pure GelMA hydrogels while hydrogels containing small amounts of HepMA displayed solely rounded morphologies (**Figure S2**). Cells that adopt a very spherical morphology are characteristic of healthy differentiated chondrocytes.^[1] These results are in line with current literature reports, further highlighting the importance of providing GelMA with additional biological cues to induce a chondrocytic morphology.^[2, 3]

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