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A Synthetic Thermosensitive Hydrogel for Cartilage Bioprinting and Its Biofunctionalization with Polysaccharides

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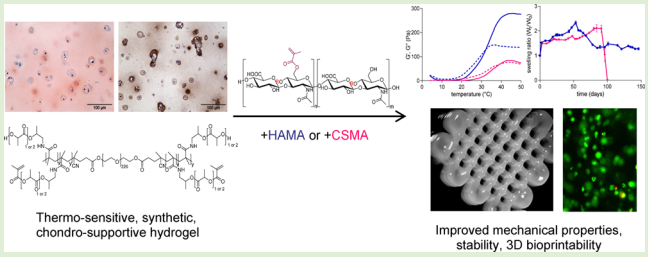
Supporting Information

ABSTRACT: Hydrogels based on triblock copolymers of polyethylene glycol and partially methacrylated poly[*N*-(2-hydroxypropyl) methacrylamide mono/dilactate] make up an attractive class of biomaterials because of their biodegradability, cytocompatibility, and tunable thermoresponsive and mechanical properties. If these properties are fine-tuned, the hydrogels can be three-dimensionally bioprinted, to generate, for instance, constructs for cartilage repair. This study investigated whether hydrogels based on the polymer mentioned above with a 10% degree of methacrylation ($M_{10}P_{10}$) support cartilage formation by chondrocytes and whether the incorporation of methacrylated chondroitin sulfate (CSMA) or methacrylated hyaluronic acid (HAMA) can improve the mechanical properties, long-term stability, and printability. Chondrocyte-laden $M_{10}P_{10}$ hydrogels were cultured for 42 days to evaluate chondrogenesis. $M_{10}P_{10}$ hydrogels with or without polysaccharides were evaluated for their mechanical properties (before and after UV photo-cross-linking), degradation kinetics, and printability. Extensive cartilage matrix production occurred in $M_{10}P_{10}$ hydrogels, highlighting their potential for cartilage repair strategies. The incorporation of polysaccharides increased the storage modulus of polymer mixtures and decreased the degradation kinetics in cross-linked hydrogels. Addition of HAMA to $M_{10}P_{10}$ hydrogels improved printability and resulted in three-dimensional constructs with excellent cell viability. Hence, this novel combination of $M_{10}P_{10}$ with HAMA forms an interesting class of hydrogels for cartilage bioprinting.



INTRODUCTION

Articular cartilage is the tissue that covers the extremities of bones inside joints. The tissue functions as a damper because of its high osmotic pressure and reduces surface friction due to its smooth surface structure. Articular cartilage contains proteoglycans, collagen type II, water, and cells, the chondrocytes. Because the tissue lacks vasculature and innervation and contains only a few chondrocytes, it has a limited regenerative capacity.[1,2] The implantation of cell-laden hydrogel scaffolds is regarded as a promising approach for the treatment of cartilage defects. Hydrogels, networks of hydrophilic polymers, have high water content, which supports cell survival and allows homogeneous encapsulation of cells as well as biological and chemical cues. Therefore, cell-laden hydrogel implants can promote new tissue formation while initially providing structural support. For the generation of successful cell-laden constructs, it is essential to have control over the mechanical properties and degradation kinetics of the construct, as it should progressively be replaced by newly formed tissue after implantation.[3] The mechanical properties and degradation kinetics of hydrogels can be easily tailored over a broad range and in a highly reproducible manner by a proper design of the building blocks.[4−6] In addition, thermoresponsive functionalities can be introduced into the building blocks, providing the opportunity to generate injectable and three-dimensional (3D) printable hydrogels.[7]

Copolymers based on a polyethylene glycol (PEG) midblock flanked by two poly[*N*-(2-hydroxypropyl) methacrylamide mono/dilactate] (polyHPMA-lac) outer blocks have recently been investigated for pharmaceutical and biomedical applications.[8−12] Methacrylated polyHPMA-lac-PEG triblock copolymers display lower-critical solution temperature (LCST)

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behavior in aqueous solutions, meaning that these polymers are soluble at low temperatures and form physical gels, by self-assembly due to dehydration of polymer chains, at temperatures above a critical temperature, called the cloud point (CP).[13] The thermosensitive behavior of methacrylated polyHPMA-lac-PEG triblock copolymers can be highly tuned, e.g., to physiologically relevant temperatures, by adapting the content of the lactate groups present in the outer blocks as well as the number of methacrylate groups.[9,13,14] In addition, the methacrylate groups allow UV light-mediated photo-cross-linking, which prevents rapid disassembly of the polymer networks.[13] Chemically cross-linked hydrogels with tailored degradation rates and mechanical properties can be obtained by varying the number of methacrylate units per polymer chain, the molecular weight of the PEG midblock and that of the thermosensitive flanking blocks, and the polymer concentration in the hydrogel.[8,9,11,13] The thermosensitive behavior of methacrylated polyHPMA-lac-PEG triblock copolymers allows easy handling of the polymer solution at low temperatures, when it behaves as a viscous liquid, to incorporate cells. Previous studies have shown a high level of viability of encapsulated articular chondrocytes in methacrylated polyHPMA-lac-PEG triblock copolymer-based hydrogels.[10] However, long-term culture and actual cartilage matrix formation in these hydrogels have not been investigated so far.

Cell-laden hydrogels can accurately be shaped with 3D biofabrication techniques to mimic the architecture of native tissues, e.g., the zonal organization of articular cartilage,[15] and to generate patient specific construct shapes. 3D bioprinting is a form of biofabrication based on computer-aided layer-by-layer material deposition.[16−19] As such, bioprinting also allows the incorporation of pores or perfusable channels into the 3D structure, for easy diffusion of nutrients, oxygen, and metabolites during (*in vitro*) construct maturation.[7] Hydrogels composed of methacrylated polyHPMA-lac-PEG triblock copolymers have already been shown to be printable because of their thermosensitive behavior.[10] However, this required a relatively high polymer concentration and a high degree of methacrylation (DM).[10] In general, dense polymer networks due to, for example, high polymer concentrations and high DM have adverse effects on the matrix production of embedded cells[20,21] and are therefore unfavorable for the fabrication of tissue repair constructs. To tackle this well-known dilemma in bioprinting,[7] hybrid materials can be designed, for example, by incorporating polysaccharides, which increase the viscosity of the polymer solution and can potentially improve the printability without hampering the matrix production of embedded cells.[22−26] In this study, the polysaccharides chondroitin sulfate (CS) and hyaluronic acid (HA) were methacrylated to allow UV photo-cross-linking[27,28] and blended with low-DM (10%) polyHPMA-lac-PEG triblock copolymers, as both are natural polysaccharides abundantly present in native cartilage. In addition, they have demonstrated anabolic effects on extracellular matrix synthesis by chondrocytes and stem cells.[24,29−34] Therefore, these polysaccharides are attractive candidates for the optimization of methacrylated polyHPMA-lac-PEG triblock copolymer-based hydrogels for cartilage bioprinting. It is hypothesized that the incorporation of methacrylated HA (HAMA) or methacrylated CS (CSMA) into methacrylated polyHPMA-lac-PEG triblock hydrogels will affect the mechanical properties, decrease the degradation rate, and improve the 3D printability in comparison to those characteristics of hydrogels made of the methacrylated polyHPMA-lac-PEG triblock only. The aim of this study was to characterize methacrylated polyHPMA-lac-PEG triblock copolymer-based hydrogels in terms of chondrogenesis, mechanical behavior, degradation kinetics, and printability. The question of whether the incorporation of HAMA or CSMA into this synthetic hydrogel can further improve the mechanical properties, affect the degradation rate, and enhance the printability was also investigated.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and all solvents from Biosolve (Valkenswaard, The Netherlands) unless indicated otherwise. Chemicals and solvents were used as received. PEG 10 kDa was supplied by Merck (Darmstadt, Germany). HA sodium salt (1560 kDa) was supplied by Lifecore Biomedical (Chaska, MN). CS A sodium salt from bovine trachea (Sigma-Aldrich) was analyzed via Viscotek gel permeation chromatography (GPC) and showed a bimodal molecular weight distribution [number-average molecular weights, M_n, of 26.9 kDa, 94% mass content, and 353.8 kDa, 6% mass content (details are given in Figure S1)]. L-Lactide was purchased from Corbion Purac (Amsterdam, The Netherlands), and Irgacure 2959 was a kind gift from BASF (Ludwigshafen, Germany). *N*-(2-Hydroxypropyl) methacrylamide (HPMA), HPMA mono- and dilactate, and PEG_{10000}-4,4′-azobis(cyanopentanoate) macroinitiator were synthesized as previously reported.[35−37] Phosphate-buffered saline (PBS), penicillin/streptomycin (pen/strep; 10000 units/mL penicillin and 10 mg/mL streptomycin), and the picogreen DNA assay were supplied by Invitrogen (Carlsbad, CA). Three different types of Dulbecco's modified Eagle's medium (DMEM) were used: DMEM 31885 from Gibco (termed DMEM), high-glucose DMEM D6429 from Sigma-Aldrich (termed high-glucose DMEM), and DMEM/F-12+GlutaMax-1 31331 from Invitrogen (termed DMEM/F-12). Fetal bovine serum (FBS) was obtained from Gibco (Invitrogen Corp.), and type II collagenase was purchased from Worthington Biochemical Corp. (Lakewood, NJ). ITS+ premix (human recombinant insulin, human transferrin, selenous acid, bovine serum albumin, and linoleic acid) was obtained from B. D. Biosciences (Breda, The Netherlands), recombinant human TGF-β1 from Peprotech (London, U.K.), Pronase (11459643001) from Roche Life Sciences (Indianapolis, IN), hyaluronidase (H2126) from Sigma-Aldrich, and Tissucol Duo S (fibrin and thrombin) from Baxter (Utrecht, The Netherlands). The antibody against collagen type I (1:100; EPR7785, ab138492) was obtained from Abcam (Cambridge, U.K.). Antibodies against collagen types II (1:100, II-6B3II) and VI (1:5, 5C6) were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Secondary horseradish peroxidase-conjugated antibodies for collagen type I (EnVision+, K4010), collagen type II (1:100, IgG HRP, P0447), and collagen type VI (EnVision+, K4007) were ordered from DAKO (Heverlee, The Netherlands). Calcein-AM (to stain living cells) and ethidium homodimer-1 (to stain nuclei of dead cells) were obtained from Life Technologies (L3224, Bleiswijk, The Netherlands). Finally, Dye-Trak 'F' microspheres (Fluorescent Orange) were ordered from Triton Technology Inc. (San Diego, CA).

Synthesis of Methacrylated Poly[*N*-(2-hydroxypropyl) methacrylamide mono/dilactate]-PEG Triblock. The synthesis of a methacrylated thermosensitive triblock copolymer, consisting of a hydrophilic PEG-based midblock flanked by two partially methacrylated pHPMA-lac outer blocks, was conducted as previously described by Vermonden et al.[13,14] Briefly, a free radical polymerization in acetonitrile was conducted at 70 °C for 40 h under a N_2 atmosphere, using PEG_{10000}-4,4′-azobis(cyanopentanoate) as a macroinitiator and HPMA mono- and dilactate (monolactate:dilactate molar ratio of 75:25) as monomers, with a monomer:macroinitiator mass ratio of 4:1. After precipitation in cold diethyl ether, the polymer was collected and further modified via partial esterification of the hydroxyl groups present on the lactate units with methacrylate groups. This reaction was conducted in dry tetrahydrofuran as a solvent, and methacrylic anhydride (MA, molar feed of 13.3% of the free hydroxyl groups of the

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polymer) was used as a methacrylating agent in the presence of triethylamine and 4-dimethylaminopyridine. The methacrylated polyHPMA-lac-PEG triblock copolymer is hereafter termed $M_{10}P_{10}$ [M_{10} refers to a DM of 10%, and P_{10} refers to a PEG block with a molecular weight (MW) of 10 kDa] and its precursor M_0P_{10}. A low DM of 10% was chosen to achieve a low network density in the cross-linked hydrogel, which is likely beneficial for cell behavior.[20]

Methacrylation of Polysaccharides. Methacrylation of CS was conducted using a transesterification reaction, as described by Abbadessa et al.[38] Briefly, CS A sodium salt was converted into tetrabutylammonium (TBA) salt (CS-TBA) by using a Dowex 50WX8 hydrogen form resin, previously saturated with TBA fluoride. Subsequently, 2.7 g (3.08 mmol of disaccharide units) of CS-TBA was dissolved in 100 mL of dry dimethyl sulfoxide (DMSO) under a N_2 atmosphere at 50 °C. Next, 4-dimethylaminopyridine (0.495 g) and glycidyl methacrylate (GMA, 195 μL) were added, and the reaction mixture was stirred at 50 °C for 48 h. After the reaction, the mixture was diluted with water and the pH was lowered to 5.5 using a 0.2 M solution of HCl in water. The polymer solution was further dialyzed against a 150 mM NaCl solution in water for 3 days and against water for 4 days. The polymer was finally collected, as Na^+ salt, after freeze-drying, and it is termed CSMA hereafter.

HA was methacrylated using a method slightly modified from that reported by Hachet et al.[28] Briefly, 0.5 g (1.25 mmol of disaccharide units) of HA was dissolved in 80 mL of ultrapure water at 4 °C overnight. Subsequently, *N*,*N*-dimethylformamide (DMF) was added to obtain a mixture with a 1:1 water:DMF volume ratio. Next, 926 μL (6.25 mmol) of MA was added dropwise at 4 °C to the HA solution while the pH was kept between 8 and 9 via the addition of 0.5 M NaOH. The pH was monitored for 4 h and adjusted to 8−9. After the mixture had been stirred overnight at 4 °C, the polymer was precipitated via the addition of NaCl (final concentration in the mixture of 0.5 M) and cold ethanol (final ethanol:water ratio of 2.3:1) and further purified by means of dialysis (molecular weight cutoff of 10000−14000 Da). Purified HAMA was collected after freeze-drying.

The DM of HAMA was investigated using a method based on the detection of methacrylic acid, which is released after basic hydrolysis of the ester bonds present in the methacrylated polysaccharide.[39] The formed methacrylic acid was detected with a high-performance liquid chromatography (HPLC) Waters 2695 separating module equipped with a Waters 2487 dual λ absorbance detector (λ = 210 nm; Waters Corp., Milford, MA) and with a C18 column (Sunfire). HAMA (15 mg) was dissolved in 10 mL of 0.02 M NaOH at 37 °C for 2 h. Subsequently, 2 mL of 2 M acetic acid was added. After filtration using a 0.2 μm filter, the samples were injected into the HPLC system and eluted at 1 mL/min using an acetonitrile/water mixture (15:85, pH 2) as the mobile phase. Calibration was performed using solutions of methacrylic acid at different concentrations in the same eluent.

Experimental Design and Hydrogel Groups. To determine if $M_{10}P_{10}$ hydrogels support chondrogenesis of chondrocytes, UV cross-linked constructs from an equine chondrocyte (passage 1; n = 3 donors)-laden $M_{10}P_{10}$ [18% (w/w)] polymer mixture were prepared. Constructs were cultured for 42 days and evaluated for evidence of chondrogenesis on days 0 (harvested directly after cell encapsulation), 28, and 42, via quantitative measurements and histology. This gel formulation is hereafter termed cell-laden hydrogel M.

To investigate whether the incorporation of HAMA or CSMA into $M_{10}P_{10}$ can improve the mechanical properties, affect the degradation rate, and enhance the printability, cell-free polymer mixtures based on $M_{10}P_{10}$ [18% (w/w)], $M_{10}P_{10}$ [14% (w/w)] blended with CSMA [4% (w/w)], and $M_{10}P_{10}$ [14% (w/w)] blended with HAMA [0.9% (w/w)] were prepared and are hereafter termed mixtures M, MCS, and MHA, respectively (Table 1). These mixtures were analyzed for their thermosensitive properties using rheological measurements. Cell-free UV cross-linked M, MCS, and MHA hydrogels were further characterized for their Young's moduli and their degradation/swelling behavior in PBS (pH 7.4) enriched with 0.02% NaN_3 at 37 °C. Finally, 3D constructs were printed with polymer mixture MHA laden with fluorescent microspheres to assess homogeneous encapsulation, using a 3D bioprinter (regenHU, Villaz-St-Pierre, Switzerland). Additionally, constructs with primary chondrocytes were printed using mixtures M, MCS, and MHA to assess viability 1 and 7 days after printing. All measurements were performed in triplicate.

Table 1. Compositions of the Three Hydrogel Groups

	polymer concentration [% (w/w)]		
hydrogel	$M_{10}P_{10}$	CSMA	HAMA
M	18	–	–
MCS	14	4	–
MHA	14	–	0.9

Chondrocyte Isolation and Culture. Primary chondrocytes were isolated from full-thickness cartilage of the stifle joints of fresh equine cadavers (n = 3; 3−10-year-old horses), with consent of the owners. Macroscopically healthy cartilage was removed from the joint under aseptic conditions, and the cartilage was digested overnight at 37 °C in DMEM supplemented with collagenase II (1.5 μg/mL), hyaluronidase (1 mg/mL), FBS (10%), and pen/strep (1%). After digestion, the cell suspension was filtered through a 40 μm cell strainer. Chondrocytes were washed with PBS and stored in liquid N_2 until further use.

To prepare cell-laden constructs, the chondrocytes were expanded in a monolayer culture for 14 days (seeding density of 5×10^3 cells/cm^2) in chondrocyte expansion medium consisting of DMEM, FBS (10%), and pen/strep (1%). The chondrocytes were harvested and mixed with the polymer mixture at passage 1 when they reached 80−90% confluence. Cell-laden constructs were cultured in chondrogenic differentiation medium consisting of high-glucose DMEM supplemented with ITS+ premix (1%), dexamethasone (0.1 μM), L-ascorbic acid 2-phosphate (0.2 mM), recombinant human TGF-β1 (10 ng/mL), and pen/strep (1%) to stimulate chondrogensis and redifferentation of the chondrocytes.[40,41]

Fabrication of Cell-Laden Chemically Cross-Linked $M_{10}P_{10}$-Based Hydrogels. $M_{10}P_{10}$ was dissolved in PBS at 4 °C, and Irgacure was added [concentration of 0.05% (w/w)]. The resulting mixture [$M_{10}P_{10}$ concentration of 20.5% (w/w)] was stirred overnight in the dark at 4 °C. The expanded chondrocytes were mixed on ice with the polymer mixture to yield a concentration of $15-20 \times 10^6$ chondrocytes/mL (concentration varied per donor). Correcting for the average weight of the added cells, we found the final concentrations of Irgacure and $M_{10}P_{10}$ in the cell-laden polymer mixture were 0.044 and 18% (w/w), respectively. The cell-laden suspension was injected into a Teflon mold, which was covered with a glass slide to generate cylindrical samples (6 mm in diameter and 2 mm in height). The filled molds were kept at 37 °C for 5 min to allow physical gelation of the hydrogel. Subsequently, chemical cross-linking was induced with a UV lamp (model CL-1000L, UVP, Cambridge, U.K.; intensity of 7.2 mW/cm^2, irradiation time of 15 min). Next, the samples were cultured at 37 °C and 5% CO_2 for 42 days in chondrogenic differentiation medium. The medium was refreshed twice a week. Fibrin gels were prepared as a positive control for cell behavior. Chondrocytes were mixed with fibrinogen (Tissucol Duo S, diluted 1:15 in PBS) to obtain a cell density of $30-40 \times 10^6$ cells/mL. Next, 30 μL of thrombin (Tissucol Duo S, diluted 1:50 in PBS, 500 IU) was pipetted into the cylindrical molds, and 30 μL of a cell-laden fibrinogen suspension was mixed into the thrombin solution to generate a final cell concentration of $15-20 \times 10^6$ chondrocytes/mL (same as for cell-laden M hydrogels). Samples were incubated for 15 min at room temperature and placed in culture with chondrogenic differentiation medium as described above.

Histology and Immunohistochemistry. On days 0 (harvested directly after cell encapsulation), 28, and 42, three samples of each hydrogel group (M and fibrin) were harvested. Part of each sample was fixed overnight in formalin (37%) and dehydrated through a graded ethanol series. After being cleared in xylene, the samples were embedded in paraffin and sectioned at a thickness of 5 μm. Sections were stained with safranin-O to visualize proteoglycans, fast green to visualize collagens, and hematoxylin to stain cell nuclei, as previously described.[42]

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Collagen types I, II, and VI were visualized via immunohistochemistry. First, the sections were deparaffinized and hydrated. Next, antigen retrieval was performed with Pronase (1 mg/mL in PBS) and hyaluronidase (10 mg/mL in PBS) for 30 min at 37 °C, followed by a 10 min blocking step with H_2O_2 (0.3% in PBS) at room temperature. The primary antibody was incubated overnight at 4 °C. Mouse IgG was used at matched concentrations for negative control staining. After incubation, the matching secondary antibody was added and incubated for 30 min for collagen type I and 60 min for collagen types II and VI, at room temperature. Finally, all stainings were visualized with a 3,3′-diaminobenzidine peroxidase substrate solution for 3−10 min and counterstained with Mayer's hematoxylin. All stained sections were evaluated and photographed using a light microscope (Olympus BX51 microscope, Olympus DP70 camera, Olympus, Hamburg, Germany).

Biochemical Assays. The remaining part of each harvested cell-laden hydrogel was weighed, freeze-dried, and weighed again to determine the sample dry weight and water content. Next, the dried hydrogels were digested overnight at 56 °C in 200 μL of papain digestion buffer [0.2 M NaH_2PO_4 and 0.01 M EDTA·$2H_2O$ in Milli-Q water (pH 6.0)] supplemented with a 250 μL/mL papain solution (16−40 units/mg of protein) and 0.01 M cysteine. To determine the glycosaminoglycan (GAG) content, as a measure of proteoglycan, a dimethylmethylene blue (DMMB)[43] assay was used with known concentrations of chondroitin sulfate C as a reference. The amount of GAG was normalized to the dry weight and DNA content of the samples, as measured by the Quant-iT PicoGreen dsDNA kit and read on a spectrofluorometer (Bio-Rad, Hercules, CA), all according to the manufacturer's protocols.

Fabrication of Chemically Cross-Linked Hydrogels Modified with Polysaccharides. Defined amounts of $M_{10}P_{10}$ and CSMA or HAMA (Table 1) were dissolved in PBS at 4 °C, and Irgacure was added as the last component [final concentration of 0.044% (w/w)]. The polymer mixture containing CSMA was stirred overnight, while the mixture containing HAMA was stirred for 48 h at 4 °C to allow complete dissolution. Subsequently, the polymer mixtures were injected into Teflon molds (sample 6 mm in diameter and 2 mm in height), incubated for 5 min at 37 °C, and UV irradiated as described for the cell-laden cross-linked M hydrogels (Fabrication of Cell-Laden Chemically Cross-Linked $M_{10}P_{10}$-Based Hydrogels). Two different hydrogel compositions, MCS and MHA, were prepared, in which $M_{10}P_{10}$ was partially replaced with CSMA and HAMA, respectively. Finally, hydrogels containing only $M_{10}P_{10}$ at the maximal total polymer concentration used for hybrid gels were prepared as a control group [18% (w/w) hydrogels M]. The total polymer concentration in MHA hydrogels was slightly lower than that in the other two hydrogels, as it was not possible to dissolve more than 0.9% (w/w) of this polysaccharide because of its high MW.

Mechanical Analysis. Thermoresponsive properties of the polymer mixtures (M, MCS, and MHA) before chemical cross-linking were studied using an AR G-2 rheometer (TA Instruments, Etten-Leur, The Netherlands), equipped with a cone−plate measuring geometry (cone diameter of 20 mm and angle of 1°). All polymer mixtures were tested under oscillation temperature sweeps from 4 to 50 °C employing a frequency of 1 Hz and a strain of 1%, which was found to be within the linear viscoelastic range of all formulations (Figure S2). Values of storage and loss moduli (G' and G'', respectively) were recorded for each sweep, and the resulting rheograms were reported showing the lines interconnecting all data points for each run.

To investigate the stiffness of hydrogel constructs after UV cross-linking, all polymer mixtures (M, MCS, and MHA) were molded as described in Fabrication of Chemically Cross-Linked Hydrogels Modified with Polysaccharides and allowed to swell for 3 h in PBS at room temperature. Next, hydrogels were examined under an unconfined compression test using a Dynamic Mechanical Analyzer (DMA) (2980 DMA, TA Instruments). The hydrogels were subjected to a preload force of 0.001 N and subsequently compressed with a force ramp rate of 0.25 N/min and an upper force limit of 1 N.[13] Young's modulus was calculated as the slope of the initial linear segment of the stress/strain curve.[22]

***In Vitro* Swelling−Degradation Study.** For all polymer mixtures (M, MCS, and MHA) cross-linked samples (6 mm in diameter, 2 mm in height, 56.5 μL in volume) prepared as described in Fabrication of Chemically Cross-Linked Hydrogels Modified with Polysaccharides were placed in glass vials (diameter of 1.75 cm) with 1 mL of PBS (pH 7.4), supplemented with 0.02% NaN_3. The vials were incubated at 37 °C, and the solutions were refreshed twice per week. At multiple time points, the hydrogels were weighed and the swelling ratio (SR) was calculated as follows:

$$\mathrm{SR} = \frac{m_{\mathrm{day}\ x}}{m_{\mathrm{day}\ 0}} \tag{1}$$

where $m_{\mathrm{day}\ x}$ represents the hydrogel mass after incubation for x days and $m_{\mathrm{day}\ 0}$ the hydrogel mass before the hydrogel was placed in PBS.

Printing of Hydrogels. A 3DDiscovery bioprinter (regenHU) equipped with a Bluepoint 4 UV lamp (point light source, wavelength range of 300−600 nm, UV-A intensity at 5 cm of 103 mW/cm^2; Hönle UV Technology AG, Gräfelfing, Germany) was used for the 3D printing of hydrogels. Filaments were generated with a micro valve (CF300H) print head, for optimal control over volume deposition rates, using optimized printer settings (Table S1). To generate porous constructs, alternating layers of vertical and horizontal filaments were deposited in the x−y plane. Cross-linking was performed in a layer-by-layer fashion, exposing each deposited layer for 3 s to UV light from a distance of 5 cm. After printing, the constructs were irradiated for an additional 9 s.

Printing of Hydrogels Loaded with Fluorescent Microspheres and Cells. To evaluate the feasibility of homogeneous cell encapsulation, polymer mixture MHA was supplemented with fluorescently labeled microspheres (Fluorescent Orange Dye-Trak 'F' microspheres, Triton Technology, diameter of 15 μm, similar to that of a single cell, concentration in the polymer mixture of 0.8 million/mL), and constructs were 3D printed using optimized print settings (Table S1). To visualize the distribution of the microspheres in the constructs, an Olympus BX51 microscope was used.

To evaluate cell viability after printing, primary chondrocytes (harvested and expanded as described in Chondrocyte Isolation and Culture) were encapsulated in mixtures M, MCS, and MHA. The cell-laden mixtures were heated to 37 °C, and three constructs were subsequently printed using the aforementioned print method reported in Printing of Hydrogels. As a positive control, cast hydrogels were prepared for each mixture using the same method that was used for the equine chondrocyte-laden hydrogels (Fabrication of Cell-Laden Chemically Cross-Linked $M_{10}P_{10}$-Based Hydrogels). Each printed construct was cut into four pieces, which were cultured in separate wells with chondrocyte expansion medium. Viability was checked on two pieces on day 1 and for the other pieces after cells had been cultured for 7 days. To check cell viability, the hydrogels were stained for 20 min with calcein-AM (4 μM in PBS) and ethidium homodimer-1 (2 μM in PBS) at 37 °C. After the constructs had been washed three times in PBS, the red and green fluorescent signals were visualized using an Olympus BX51 microscope, and three images of each hydrogel quarter were analyzed.

Statistics. Statistical analyses were performed using SPSS software (version 20, IBM Corp.). Differences in Young's modulus among the hydrogel groups (M, MHA, and MCS) and differences in chondrocyte viability after printing at each time point were determined with a one-way analysis of variance (ANOVA) test. For GAG values normalized to the DNA content, both hydrogels (M and fib) at all time points (six groups in total) were compared with each other using a randomized block design ANOVA to correct for donor variability. The GAG, DNA, and water contents normalized to the dry weight at the different time points were compared to each other within each hydrogel formulation by a randomized block design ANOVA. A significance level of 0.05 and a Tukey's post hoc analysis were used for all tests.

RESULTS AND DISCUSSION

Synthesis and Characterization of Thermosensitive Polymers and Methacrylated Polysaccharides. M_0P_{10} and

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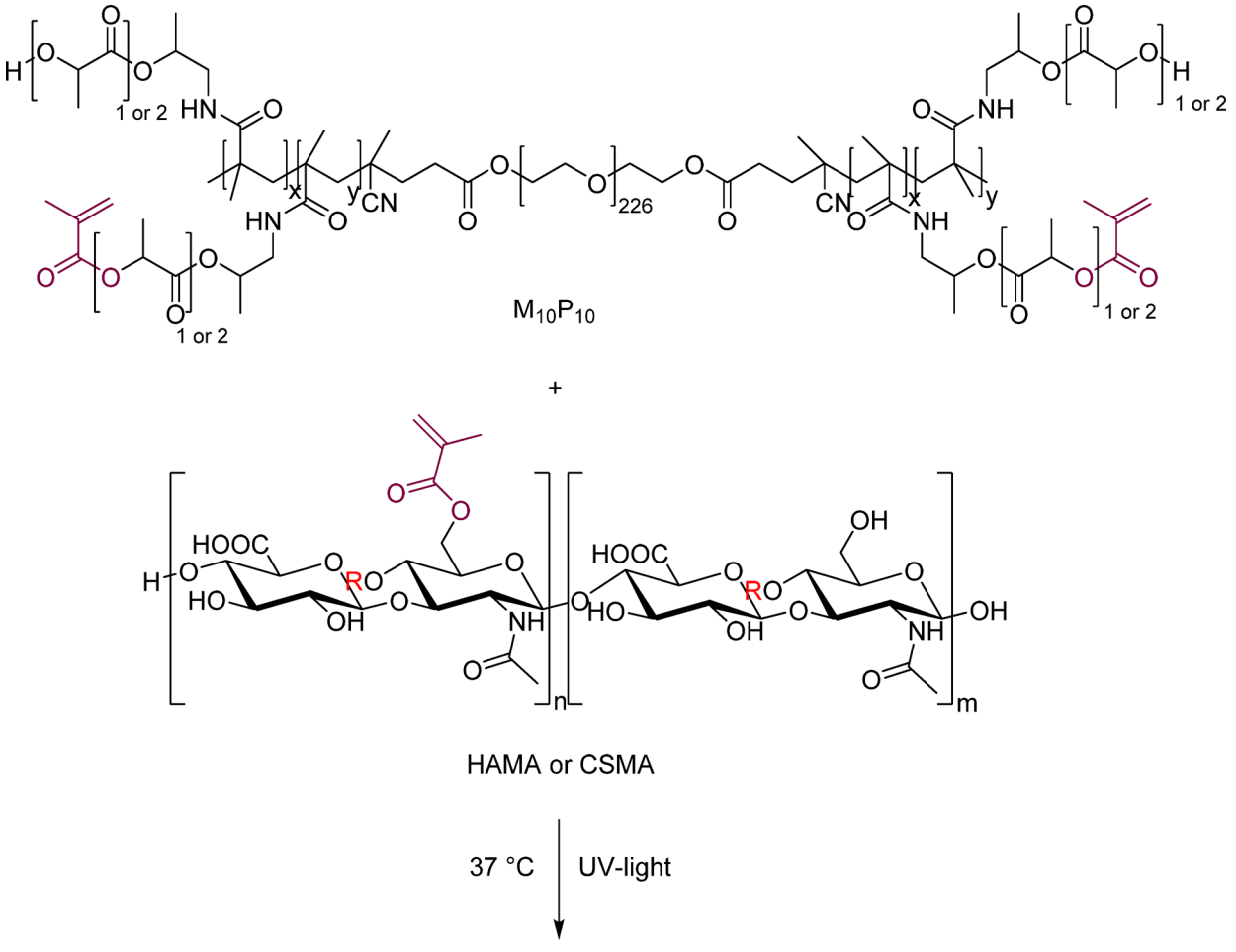

Figure 1. Chemical structure of $M_{10}P_{10}$ (top) and methacrylated HA (bottom; R = H in the equatorial position) or CS (bottom; R = SO_3H in the axial position). $M_{10}P_{10}$ confers thermosensitive properties to the gel, whereas the presence of methacrylate groups in both polymers allows UV-mediated chemical cross-linking.

$M_{10}P_{10}$ (Figure 1) were obtained in high yields (80 and 96%, respectively). Their chemical structures, confirmed by ^{1}H nuclear magnetic resonance (NMR), were in accord with previously reported data.[13,14] The M_n and DM of $M_{10}P_{10}$ determined by ^{1}H NMR were 42.4 kDa and 10.7%, respectively, whereas the M_n according to GPC was 34.6 kDa with a PDI value of 2.0. The cloud points of M_0P_{10} and $M_{10}P_{10}$ were 35 and 20 °C, respectively. Table 2 summarizes the polymer characteristics of M_0P_{10} and $M_{10}P_{10}$.

The methods employed for the methacrylation of CS and HA resulted in high yields of CSMA and HAMA (>84% for both polysaccharides). The methacrylated polysaccharides (chemical structures shown in Figure 1) were analyzed by ^{1}H NMR. The presence of the signals at 6.2 and 5.8 ppm, representative of the two vinyl protons present in the methacrylate groups, and the signal at 2.0 ppm, typical of the protons belonging to its methyl group, confirmed the partial functionalization of the hydroxyl groups with methacrylate groups.

The methacrylation of CS was performed in DMSO using GMA as a methacrylating agent, and a molar feed of GMA and CS-TBA repeating units of 0.48:1 resulted in a DM of 15.2% (Table 2), calculated according to ^{1}H NMR. Moreover, the absence in the ^{1}H NMR spectrum of the signals at 5.5 and 5.2 ppm representative of a possible glyceryl spacer between the methacrylate group and the disaccharide unit excluded the presence of products originating from ring opening reaction.[44] Thus, the reaction mechanism follows a transesterification mechanism, which is in line with our previous findings.[38]

Table 2. Characteristics of Thermosensitive Polymers and Polysaccharides

polymer	DM (%)	M_n (kDa)	PDI	CP (°C)
M_0P_{10}	0[a]	43.9[a]		35[c]
		36.2[b]	1.9[b]	
$M_{10}P_{10}$	10.7[a]	42.4[a]		20[c]
		34.6[b]	2.0[b]	
CS	0[a]	26.9 (94%)[d]	1.4[d]	na[h]
		353.8 (6%)[d]	1.3[d]	
CSMA	15.2[a]	nd[g]	nd[g]	na[h]
HA	0[a]	1560[e]	nd[g]	na[h]
HAMA	23.4[f]	nd[g]	nd[g]	na[h]

[a]Determined by ^{1}H NMR. [b]Determined by GPC. [c]Determined by UV−vis spectrophotometry. [d]Determined by Viscotek. [e]Average MW determined by multiangle light scattering−size exclusion chromatography (MALS−SEC) as described by the supplier. [f]Determined by HPLC. [g]Not determined. [h]Not applicable.

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For the synthesis of HAMA, we selected the method reported by Hachet et al.[28] This reaction was performed in a mixture of water and DMF using a large excess of MA (molar ratio of 5:1 between MA and repeating units of HA). This high feed ratio is generally used for methacrylation reactions in an aqueous environment because it is necessary to compensate for the amount of MA lost as methacrylic acid due to hydrolysis.[45,46] A lower polymer concentration, 3.1 mg/mL instead of 12.0 mg/mL, was used compared to that used in previously reported reactions, which were performed using a lower-MW HA.[28,47] The use of a relatively low concentration was necessary to facilitate pH monitoring and general handling of the reaction mixture, considering the high viscosity of high-MW HA solutions. This low HA concentration likely explains our lower level of methacrylate incorporation (5%) compared with the values from previous reports (≥14%).[28,47] Because of the poor resolution of the ^{1}H NMR spectra for high-MW HAMA, an HPLC-based method was employed to accurately determine the DM, which was found to be 23.4% (Table 2).

Matrix Production of Embedded Chondrocytes. Hydrogels composed of methacrylated polyHPMA-lac-PEG triblock copolymers have been shown to support the short-term survival of chondrocytes; however, the effect on matrix production has not been reported.[10] In this study, equine chondrocytes were encapsulated into an 18% $M_{10}P_{10}$-based hydrogel (hydrogel M) and cultured for up to 42 days in chondrogenic differentiation medium. The matrix production in this hydrogel was compared to that of chondrocytes embedded in a fibrin gel (positive control), which is gold standard for clinical delivery of cells for cartilage repair procedures and is known to support chondrogenesis because of its bioactive peptide sequences.[48,49] Hydrogel M supported cartilage-like tissue formation of the encapsulated chondrocyte, and safranin-O staining revealed a homogeneous deposition of proteoglycans after cells-laden constructs had been cultured for 28 and 42 days (Figure 2). In addition, immunolocalization of collagen type II revealed that its deposition was limited to distinct areas around the cells on day 28. However, after 42 days, a more homogeneous distribution was observed. Both stainings were more intense in the fibrin gels on days 28 and 42 than in hydrogel M samples at these time points (Figure 2). An explanation for this effect is the compaction of the fibrin gels during the first days of culture.[50−52] Because of this, the relative cell density and amount of matrix per gel volume increased as can be observed in the high DNA/dry weight and GAG/dry weight values for fibrin samples (Figure 3e,f). The sample dry weight was 10 times higher for hydrogels M than for fibrin gels, and this difference persisted over time (data not shown). Water volume normalized to the dry weight of M hydrogels increased on days 28 and 42 compared to that on day 0 (Figure 3d, 250 and 330%, respectively). Although hydrogel compaction after implantation in a defect may localize the cells at the bottom of the defect, it will on the other hand result in an incomplete defect fill. Moreover, contracting materials may be difficult to combine in hybrid scaffolds, e.g., hydrogel constructs reinforced with polymeric fibers, aimed to increase construct stiffness.[7,22,53] In these hydrid constructs, shrinking is a major drawback because it may cause stress at the interface and lead to a loss of construct integrity.

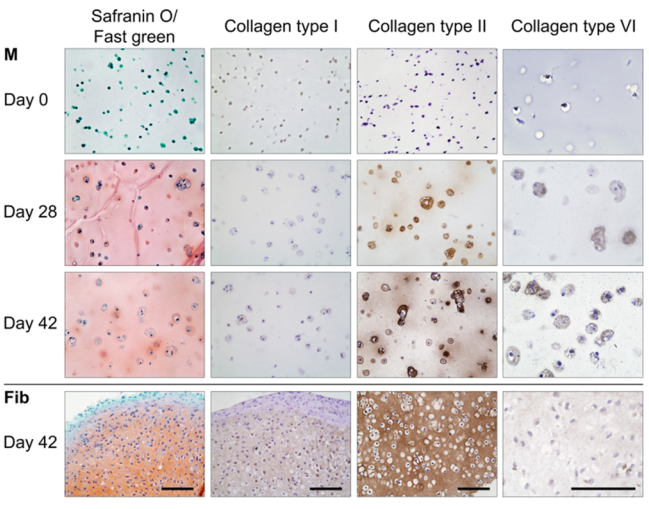

Figure 2. Histology and immunohistochemistry of chondrocytes differentiated in $M_{10}P_{10}$-based hydrogels (M) with fibrin (fib) as a positive control. From left to right: safranin-O staining and collagen type I, II, and VI staining, respectively. Scale bars represent 100 μm and are the same for all images of the same staining (column).

Collagen type VI staining was performed to visualize chondron formation. Chondrons are chondrocytes with their pericellular matrix, consisting of proteoglycans and collagen types II and VI,[54] and are known to be more active in matrix deposition than chondrocytes.[55] In hydrogels M, collagen type VI positive areas were found around the cells after they had been cultured for 28 and 42 days, indicating that chondrocytes formed chondron-like structures during culture. In fibrin samples, a slight overall positive collagen type VI staining was found. Further, only limited positive staining for collagen type I was observed in all hydrogel samples, suggesting limited dedifferentiation of the embedded chondrocytes.

Quantitative measurements were performed for GAG, DNA, and water content. However, a large variation in cell performance of the three different equine donors (3−10 years old) was observed (Figure 3), which is in line with the results of previous studies.[56] GAG content normalized to DNA content (GAG/DNA) was similar in M hydrogels on days 28 and 42 [27 ± 9 and 26 ± 10 μg/μg, respectively (Figure 3a)]. On day 28, GAG/DNA was statistically higher than the fibrin control gels [16 ± 6 μg/μg (Figure 3a)] at this time point. After a 42 day culture period, both hydrogel formulations performed equally. The GAG content normalized to the dry weight of both the M and fibrin hydrogels increased with time (Figure 3b,e). However, DNA levels normalized to the dry weight showed a significant increase for only the M hydrogels over time [0.52 ± 0.18 μg/mg on day 0 and 0.81 ± 0.30 μg/mg on day 42 (Figure 3c)], indicating cell proliferation. Finally, higher GAG/dry weight and DNA/dry weight values were found for fibrin gels than for hydrogels with formulation M, which can be explained by the compaction and relatively fast degradation of the fibrin gels. In addition, M hydrogels seemed to swell during cultures as the H_2O/dry weight increased during culture.

Thus, chondrocytes in hydrogels with formulation M produced similar levels of cartilage-like matrix compared to that of chondrocytes in fibrin gels. In addition, no compaction occurred for M hydrogels. Encouraged by these results, we further evaluated hydrogels with formulation M and incorporated CSMA and HAMA to optimize the mechanical properties, degradation kinetics, and printability.

Thermogelation of Polymer Mixtures before Chemical Cross-Linking. Figure 4 shows storage and loss moduli,

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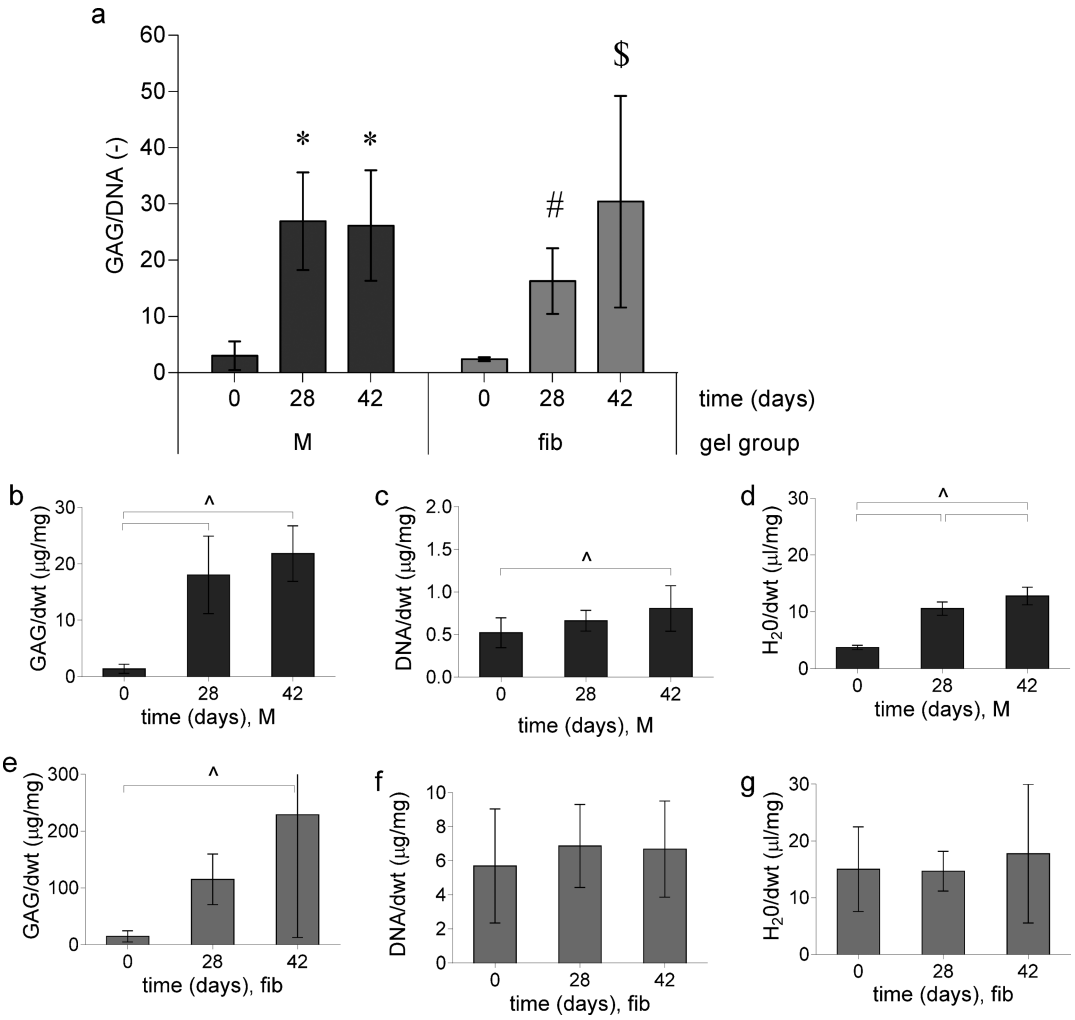

Figure 3. Quantitative GAG, DNA, and water measurements for equine chondrocytes encapsulated in $M_{10}P_{10}$-based hydrogels (M) and fibrin (fib) gels. (a) GAG content normalized to DNA for both hydrogels over time. An asterisk denotes significant differences compared to day 0. A number sign denotes that the group is significantly higher than the day 0 controls but lower compared to fibrin on day 42. A dollar sign denotes that the group is significantly higher than the day 0 controls and day 28 fibrin samples but equal to the M hydrogels on days 28 and 42. (b–d) GAG, DNA, and water content, respectively, normalized to the dry weight (dwt) for M hydrogels over time. (e–g) GAG, DNA, and water content, respectively, normalized to the dry weight (dwt) for fibrin gels over time. A caret denotes a significant difference between groups.

G' and G'', respectively, as a function of temperature for all polymer mixtures. Mixtures based only on $M_{10}P_{10}$ exhibited an increase in G' with an increase in temperature, up to 29 ± 2 Pa at 50 °C, while G'' displayed higher values over the whole temperature range (Figure 4a). $M_{10}P_{10}$ is a thermosensitive polymer capable of self-assembling and forming hydrophobic domains above defined temperatures, leading to a physical gel within a certain range of concentrations.[13] The absence of a gelation temperature (T_{gel}), here defined as the temperature at which G' crosses G'', as well as the low value of G' reached with an increase in temperature for polymer mixture M, is due to the relatively low concentration and high CP (20 °C) of the thermosensitive polymer used in this study.

Panels b and c of Figure 4 show that a continuous increase in G' as a function of temperature was observed for aqueous systems of MCS and MHA. The values of the storage modulus at 37 and 50 °C were 56 ± 6 and 84 ± 24 Pa, respectively, for MCS hydrogels and 216 ± 14 and 263 ± 12 Pa, respectively, for MHA hydrogels. For both MCS and MHA mixtures, a T_{gel} was found (39 °C for MCS hydrogels and 32 °C for MHA hydrogels). In line with previous findings, it can be observed that the partial replacement of $M_{10}P_{10}$ with CSMA or HAMA resulted in the formation of physical gels with G' values above 20 °C much higher than those of polymer mixtures composed of only $M_{10}P_{10}$.[38] The beneficial role of the added polysaccharide in terms of the mechanical properties of the hydrogel is more remarkable for MHA hydrogels, where an even lower total polymer concentration (Table 1) led to the formation of the stiffest hydrogel (G' = 216 ± 14 at 37 °C). The rheological behavior of the polysaccharide-enriched formulations clearly shows that the elastic properties of hydrogels based on $M_{10}P_{10}$ can be improved by the addition of polysaccharides, without increasing the total polymer concentration.

Mechanical Properties and *in Vitro* Swelling–Degradation Behavior of Chemically Cross-Linked Hydrogels. The injection of polymer mixtures into a Teflon mold at 4 °C, followed by a temperature increase to 37 °C and UV irradiation for 15 min, resulted in the formation of cylindrically shaped constructs. Figure 5 shows Young's moduli for the different hydrogel constructs after samples had swelled in PBS for 3 h. The values of Young's modulus were 13.7 ± 1.1, 16.0 ± 1.4,

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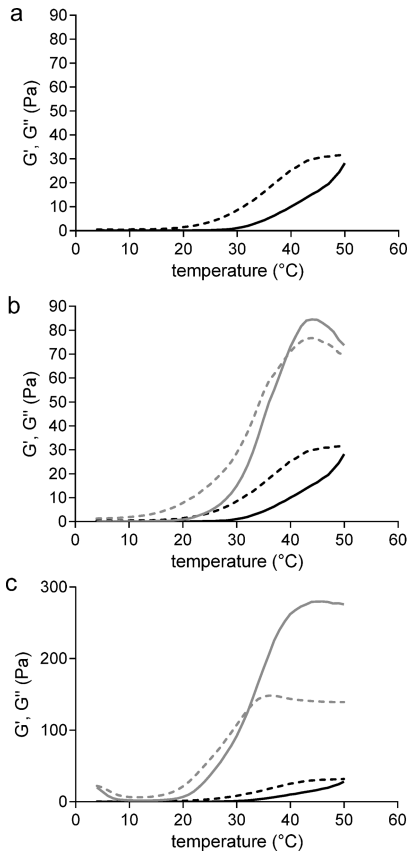

Figure 4. Rheograms of polymer mixtures. G' (—) and G'' (---) moduli as a function of temperature, recorded during a temperature sweep experiment from 4 to 50 °C for (a) hydrogels based on 18% (w/w) $M_{10}P_{10}$ (M hydrogels), (b) hydrogels based on 14% (w/w) $M_{10}P_{10}$ and 4% (w/w) CSMA (MCS hydrogels, gray lines) compared with M hydrogels (black lines), and (c) hydrogels based on 14% (w/w) $M_{10}P_{10}$ and 0.9% (w/w) HAMA (MHA hydrogels, gray lines) compared with M hydrogels (black lines).

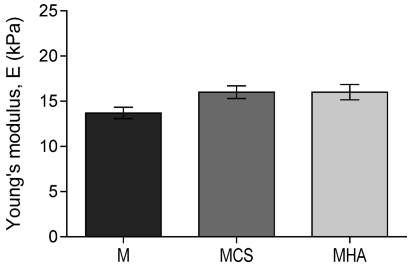

Figure 5. Dynamic mechanical analysis of chemically cross-linked hydrogels. Young's moduli for hydrogels based on $M_{10}P_{10}$ (M), hydrogels based on $M_{10}P_{10}$ and CSMA (MCS), and hydrogels based on $M_{10}P_{10}$ and HAMA (MHA), measured under unconfined compression (n = 3).

and 16.0 ± 1.9 kPa for M, MCS, and MHA hydrogels, respectively. No significant differences among the three hydrogel formulations were found. Hence, no differences in the cell response due to different mechanical stimuli can be expected in the three hydrogels. The influence of polysaccharide molecular weight on the final stiffness can be illustrated by comparing MCS and MHA hydrogels. Hydrogels with comparable Young's moduli were obtained, despite the much lower concentration of the higher-MW polysaccharide (0.9% vs 4%) and the smaller number of methacrylate groups in MHA hydrogels, calculated considering the slight difference in the DM of the two polysaccharides (Figure 5). In line with our findings, the positive influence of HA with a higher MW has been reported previously for hybrid hydrogel systems based on acrylated HA and thiol-modified four-arm PEG or thiol derivatives of HA and PEG-vinylsulfones, cross-linked via Michael addition-type reaction.[57,58] As can be expected for hydrogel materials, the stiffness of these hydrogel constructs is significantly lower than that of native cartilage (400−800 kPa[59−61]).

Figure 6 shows that M hydrogels initially swelled for 38 days, during which the SR reached a maximum of 2.3 ± 0.1.

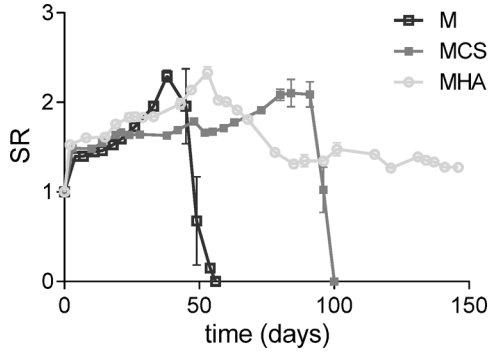

Figure 6. Swelling and degradation profiles for hydrogels based on $M_{10}P_{10}$ (M), hydrogels based on $M_{10}P_{10}$ and CSMA (MCS), and hydrogels based on $M_{10}P_{10}$ and HAMA (MHA) in PBS buffer at 37 °C. Error bars represent the standard deviation of experiments performed in triplicate. SR represents the swelling ratio and was calculated according to eq 1.

Complete degradation occurred after incubation at 37 °C for 56 days. This degradation profile is in line with previously reported studies.[8,13] The degradability of hydrogels based on chemically cross-linked polyHPMA-lac-PEG triblock copolymers at pH 7.4 and 37 °C is due to the hydrolysis of several ester bonds.[9] The first soluble degradation products are lactic acid units obtained by the hydrolysis of OH-terminated lactate side chains. Consequently, the remaining gel matrix exhibits an increased hydrophilic character with a higher water uptake capacity, leading to the typical swelling phase. Mass loss is seen when the elimination of the water-soluble degradation products from the matrix exceeds the water uptake. This swelling−degradation behavior might also explain the absence of an increase in the level of GAG in the chondrocyte-laden M hydrogels between culture days 28 and 42. The swelling process and the presence of a partially degraded and thus less dense hydrogel matrix between days 28 and 42 may have contributed to the leaching of newly formed GAGs out of the gel.[62]

In contrast to M hydrogels, the hydrogels containing polysaccharides degraded much slower (Figure 6). More specifically, MCS hydrogels swelled for 91 days with a maximal SR of 2.1 ± 0.2 and underwent complete disintegration in 100 days, whereas the degradation profile of MHA hydrogels showed a maximal SR of 2.3 ± 0.1 on day 53, followed by partial mass loss during the subsequent 32 days, that reached a plateau in SR of 1.4 for the subsequent 61 days of monitoring. Thus, the presence of the two polysaccharides increased the stability of the hydrogels under the tested conditions. In fact, the loss of polysaccharides from these hydrogels can only occur after the polysaccharide molecules diffuse out of the hydrogel matrix and are dissolved in the surrounding buffer. This

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phenomenon can take place only after complete hydrolysis of the ester bonds of the polymerized methacrylate groups, which connect a polysaccharide chain to another polysaccharide or $M_{10}P_{10}$ chain. However, it has been reported that polymerized methacrylate groups directly attached to polysaccharide chains are very stable at pH 7.4 and 37 °C.[63,64] Therefore, it was not surprising that no complete degradation of MHA hydrogels was observed under the applied conditions. Keeping this in mind, we can ascribe the full mass loss observed for MCS hydrogels after 100 days to disintegration of the macroscopic hydrogel in smaller fragments, which is confirmed by the observation that the PBS buffer was slightly turbid during the last days of the study.

In general, the highest stability of the hydrogels is observed when $M_{10}P_{10}$ is partially replaced with HAMA (MHA hydrogels) at the tested concentrations. Nevertheless, the degradation profile of the polysaccharide-enriched hydrogels would likely be different if they were tested *in vivo*, because of the role played by enzymatic degradation via, e.g., hyaluronidase, should be taken into consideration.[65]

Three-Dimensional Printing of Hydrogels. Shape stable, 3D-printed hydrogel constructs with highly regular internal porosity were obtained, when printing MHA hydrogels, above the T_{gel} (Figure 7a–c). Polymer mixtures M and MSC could not be printed with high shape fidelity at cell friendly temperatures, as polymer mixture M did not form a stable physical gel below 40 °C and the MCS polymer mixture had an overly low viscosity at 37 °C, forming only a weak physical gel at cell friendly temperatures.

In line with previous observations, polymer mixtures exhibiting physical hydrogel formation and a relatively high G' (216 ± 14 Pa) at 37 °C allowed adequate stability of the extruded filaments on the deposition plate (preheated at 40 °C), and thus 3D printing with high shape fidelity (MHA hydrogels).[38] On the other hand, the rheological properties of the MCS polymer mixture were found to be insufficient for successful 3D printing.

Fluorescent microbeads with sizes similar to those of cells (diameter of 15 μm) were homogeneously dispersed in the MHA polymer mixture before printing. This homogeneous distribution was maintained during the printing process (Figure 7d). To investigate the influence of printing on cell viability, primary chondrocytes were dispersed in the three polymer mixtures (M, MCS, and MHA) and 3D constructs were printed. Cell viability was found to be between 85 and 95%, both 1 and 7 days after printing, similar to those of the cast hydrogel controls (Figure 7e), indicating good biocompatibility for all three hydrogel formulations and no adverse effects caused by the printing procedure.

In a previous study, a hydrogel based on cross-linkable pHPMA-lac-PEG triblock copolymers was used to print porous 3D structures. However, this required a relatively high polymer concentration [25% (w/w)] and DM (30%).[10] The addition of HAMA has led to a hydrogel platform that could be printed at a considerably lower concentration (14% $M_{10}P_{10}$ and 0.9% HAMA) and a DM of the thermosensitive polymer (10%), which is likely beneficial for the cartilage-like matrix deposition of incorporated cells.[20,21] In addition, the presence of HAMA itself is likely to improve the cartilage-like tissue production and remodeling by embedded chondrocytes.[23,24,29−34,66] In fact, the differentiation potential of chondrocytes in hydrogels with formulation MHA (and MCS) was confirmed by collagen type II detection after a 42 day culture (Figure S3). Nevertheless,

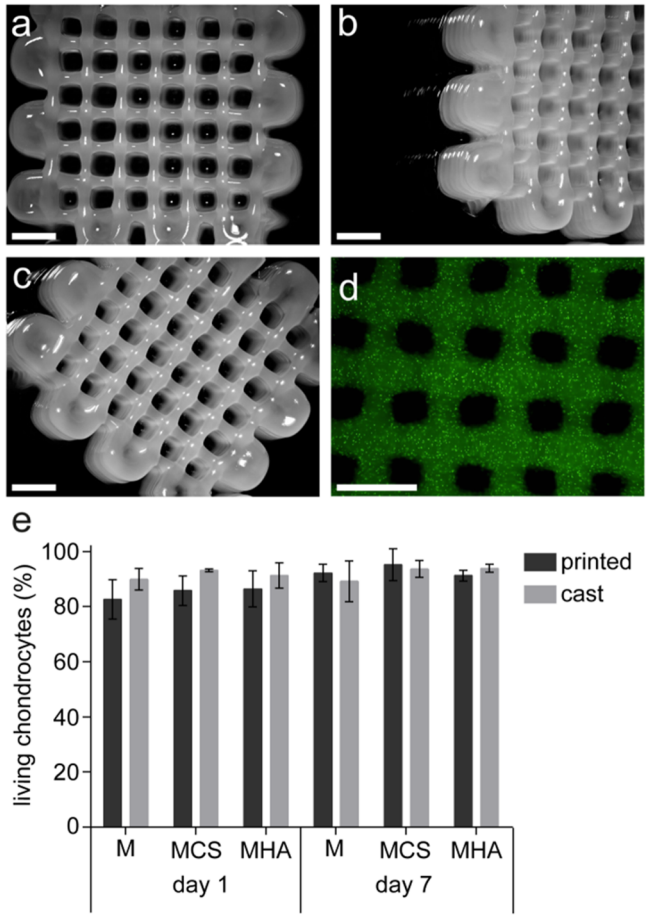

Figure 7. 3D-printed porous constructs based on MHA: (a) top view, (b) top−side view, (c) top−corner view, and (d) top view showing a homogeneous distribution of encapsulated green fluorescent beads. (e) Percentage of living chondrocytes in printed and cast (control) constructs for each hydrogel formulation after cells had been cultured for 1 and 7 days. No statistical differences were observed between hydrogel formulations. The scale bar represents 2 mm.

the exact concentration of HAMA still needs further attention with respect to this aspect, as studies have reported a dose-dependent effect in which high HA(MA) concentrations have a less stimulating effect or produce even a reduction in the level of cartilage-like tissue formation of chondrocytes compared to a lower HA(MA) concentration.[24,67−71] Taken together, the partial replacement of the pHPMA-lac-PEG triblock copolymer with a small amount of HAMA, in combination with a layer-by-layer UV irradiation strategy during the printing process, is a promising approach for cell friendly additive manufacturing of these hydrogels.

CONCLUSIONS

In this study, UV cross-linked hydrogels based on thermosensitive methacrylated pHPMA-lac-PEG triblock copolymer, laden with equine chondrocytes, showed potential for significant cartilage-like tissue formation *in vitro*. Additionally, mechanical analysis and swelling−degradation studies proved that the partial replacement of the methacrylated pHPMA-lac-PEG triblock copolymer with CSMA or HAMA can lead to the design of hydrogels with an improved thermosensitive profile, a similar stiffness after UV cross-linking, and a slower degradation rate compared to those of hydrogels consisting of only

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pHPMA-lac-PEG triblock copolymers. Moreover, hydrogels containing HAMA (MHA hydrogels) were used to 3D bioprint porous structures without adversely affecting cell viability. Taken together, MHA hydrogels are attractive systems for the design of 3D cell-laden constructs for cartilage regeneration.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.6b00366.

GPC characterization of chondroitin sulfate (CS), identification of the linear viscoelastic range (LVR) of polymer mixtures, differentiation potential of chondrocytes in MCS and MHA hydrogels, and input parameters for creating 3D-printed constructs (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

3D, three-dimensional; CP, cloud point; CS, chondroitin sulfate; CSMA, methacrylated chondroitin sulfate; CS-TBA, chondroitin sulfate tetrabutylammonium salt; DM, degree of methacrylation; DMA, dynamic mechanical analyzer; DMEM, Dulbecco's modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; DMMB, dimethyl methylene blue; DMSO, dimethyl sulfoxide; dwt, dry weight; FBS, fetal bovine serum; GAG, glycosaminoglycan; GMA, glycidyl methacrylate; GPC, gel permeation chromatography; HA, hyaluronic acid; HAMA, methacrylated hyaluronic acid; HPLC, high-performance liquid chromatography; HPMA, *N*-(2-hydroxypropyl) methacrylamide; LCST, lower critical solution temperature; M, polymer mixture and hydrogel composed of 18% (w/w) $M_{10}P_{10}$; M_0P_{10}, non methacrylated polyHPMA-lac-PEG triblock copolymer; $M_{10}P_{10}$, partially methacrylated polyHPMA-lac-PEG triblock copolymer; MA, methacrylic anhydride; MCS, polymer mixture and hydrogel composed of 14% (w/w) $M_{10}P_{10}$ and 4% (w/w) CSMA; MHA, polymer mixture and hydrogel composed of 14% (w/w) $M_{10}P_{10}$ and 0.9% (w/w) HAMA; M_n, number-average molecular weight; MW, molecular weight; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; pen/strep, penicillin/streptomycin; PDI, polydispersity index; PEG, polyethylene glycol; polyHPMA-lac, poly[*N*-(2-hydroxypropyl) methacrylamide mono/dilactate]; SR, swelling ratio; TBA, tetrabutylammonium; T_{gel}, gelation temperature

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