Hydrolytically Degradable Hyaluronic Acid Hydrogels with Controlled Temporal Structures

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Polysaccharides are being processed into biomaterials for numerous biological applications due to their native source in numerous tissues and biological functions. For instance, hyaluronic acid (HA) is found abundantly in the body, interacts with cells through surface receptors, and can regulate cellular behavior (e.g., proliferation, migration). HA was previously modified with reactive groups to form hydrogels that are degraded by hyaluronidases, either added exogenously or produced by cells. However, these hydrogels may be inhibitory and their applications are limited if the appropriate enzymes are not present. Here, for the first time, we synthesized HA macromers and hydrogels that are both hydrolytically (via ester group hydrolysis) and enzymatically degradable. The hydrogel degradation and growth factor release was tailored through the hydrogel cross-linking density (i.e., macromer concentration) and copolymerization with purely enzymatically degradable macromers. When mesenchymal stem cells (MSCs) were encapsulated in the hydrogels, cellular organization and tissue distribution was influenced by the copolymer concentration. Importantly, the distribution of released extracellular matrix molecules (e.g., chondroitin sulfate) was improved with increasing amounts of the hydrolytically degradable component. Overall, this new macromer allows for enhanced control over the structural evolution of the HA hydrogels toward applications as biomaterials.

Introduction

HA is a linear polysaccharide of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine, found natively in many tissues (e.g., cartilage),[1–3] and degrades primarily by hyaluronidases found throughout the body or through oxidative mechanisms to yield oligosaccharides and glucuronic acid.[4] HA plays a role in cellular processes like cell proliferation, morphogenesis, inflammation, and wound repair and interacts with cells through surface receptors (CD44, CD54, and CD168).[1,2,5,6] These biological interactions make HA a candidate for the development of biomaterials that can directly interact with cells.

Importantly, HA can be readily modified through its carboxyl and hydroxyl groups to form hydrogels in the presence of water.[7–13] These hydrogels have found numerous applications in tissue regeneration,[8,13–15] drug delivery,[12] and microdevices.[16] However, the design of these current hydrogels is limiting in that (i) enzymes are needed to degrade the hydrogel, which can hinder the diffusion of growth factors, migration of cells, and distribution of extracellular matrix proteins if enzymes are not abundant and (ii) degradation products are typically modified forms of HA (e.g., due to methacrylate addition) rather than potentially biologically active unmodified HA.[17–20] Although an ester group may exist between the HA backbone and the reactive group, this bond is typically very stable, potentially due to steric hindrance or the hydrophobicity of the surrounding chemical groups.

These limitations motivated our work in designing the next generation of HA hydrogels with superior properties. Specifically, we sought to design a new macromer that forms hydrogels that are hydrolytically degradable to allow further control over their structures toward a range of biological applications. These macromers can be polymerized into hydrogels alone or copolymerized with other macromers to produce hydrogels with diverse properties, specifically related to temporal structures with degradation. Here, we report the synthesis of the novel HA macromer (MeLAHA) and illustrate potential diversity in applications through the release of growth factors and interactions with cells.

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Experimental Section

Macromer Syntheses. A detailed description of the MeLAHA macromer synthesis can be found in the Supporting Information and the reaction is illustrated in Scheme 1. Briefly, 2-hydroxyethyl methacrylate (HEMA; 0.0549 mmol, Acros organics) is reacted with DL-lactide (0.0823 mmol, Polysciences) via a ring opening polymerization (110 °C, 1 h, under nitrogen) in the presence of stannous octoate (0.0004 mmol, Sigma) to obtain MeLA-OH. The end group is converted into a carboxylic acid through reaction (50 °C, 24 h, under nitrogen) of MeLA-OH (0.024 mmol) with succinic anhydride (0.024 mmol, Sigma) in the presence of pyridine (Sigma) and dimethylaminopyridine (DMAP; 0.002 mmol, Sigma) to obtain MeLA-COOH. The end group of the MeLA-COOH (0.008 mmol) is functionalized by reacting (0 °C, 4 h and room temperature 24 h) with *N*-hydroxysuccinimide (NHS; 0.008 mmol, Sigma) and dicyclohexylcarbodiimide DCC; 0.008 mmol, Acros organics) to obtain MeLA-NHS. The sodium salt of HA (Lifecore, 64 kDa) is converted to a tetrabutylammonium (TBA; Sigma) salt by acidic ion exchange (room temperature, 8 h) with Dowex 50 W × 8–200 resin, neutralized in aqueous TBA hydroxide for solubilization in anhydrous dimethyl sulfoxide (DMSO), coupled with MeLA-NHS (40 °C, 24 h), and purified through precipitation in acetone to obtain MeLAHA.

Each product was confirmed by ^{1}H NMR (Bruker DMX 360 and 300 MHz spectrometer) and stored in acetone until use. Diffusion-ordered NMR Spectroscopy (DOSY) spectra were recorded using stimulated echo pulse sequence with bipolar gradients and a longitudinal eddy current delay in a Bruker DMX 600 MHz NMR spectrometer having *z*-gradient (maximum strength of 70 G/cm). The sine-shaped gradient pulse with 3.0 ms duration was logarithimically incremented

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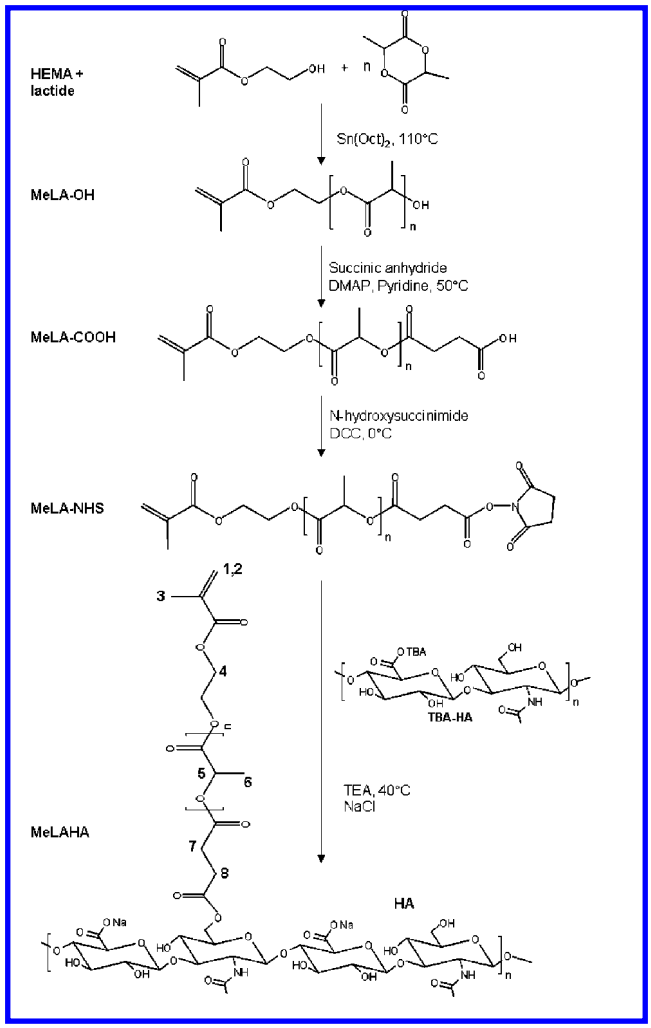
Scheme 1. Synthesis of MeLAHA Macromer[a]



[a] HEMA = 2-hydroxyethyl methacrylate, Me = methacrylate, LA = lactic acid, DMAP = dimethylaminopyridine, DCC = dicyclohexylcarbodiimide, NHS = *N*-hydroxysuccinimide, HA = hyaluronic acid, TBA = tetrabutylammonium, TEA = triethylamine.

in 32 steps, from 2% up to 95% of the maximum gradient strength. Diffusion time was set to 300 ms and a longitudinal eddy current delay of 5 ms was used. After Fourier transformation and baseline correction, DOSY spectra were processed using Bruker Topspin software's DOSY package.

MeHA was synthesized by the addition of methacrylic anhydride (~20-fold excess, Sigma) to a solution of 1 wt% HA (Lifecore, 64 kDa) in deionized water adjusted to a pH of 8 with 5 N NaOH and reacted on ice for 24 h.[7,21] For purification, the macromer solution was dialyzed (MW cutoff 5–8 kDa) against deionized water for at least 48 h and the final product was obtained by lyophilization. ^{1}H NMR was used to determine the final functionality and purity of the methacrylated HA (MeHA).

Hydrogel Formation. Hydrogels were synthesized by dissolving the MeLAHA or MeHA macromers at various concentrations (~ 1, 2, and 4 wt%) in phosphate buffered saline (PBS). The photoinitiator, 2-methyl-1-[4-hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959) was added at 0.05 wt% and the macromer solution was placed into a mold (5 mm diameter, 2 mm thick) and polymerized with ~2mW/cm^2 ultraviolet light (~365 nm, EIKO bulb) for 10 min. For growth factor release studies, human vascular endothelial growth factor (VEGF; R&D Systems) was added to the macromer solutions prior to photopolymerization.

Hydrogel Degradation and Growth Factor Release. After polymerization, the hydrogels ($n = 3$ per composition) were placed in separate wells of a 24-well plate containing 1 mL of PBS and placed on an orbital shaker. The PBS containing the released VEGF and degradation products was removed at various time points and stored at −20 °C. The amount of uronic acid (a degradation component of HA) released during degradation was measured using a previously established carbazole reaction technique,[22] and the VEGF concentration was determined in all of the samples using DuoSet ELISA development kits (R&D Systems) and reported as the fraction of the cumulative amount released with degradation.

Cellular Interactions with HA Hydrogels. Bone marrow-derived human MSCs (Lonza) were expanded in growth media (α-MEM, 16.7% FBS, 1% penicillin/streptomycin (PS), and 2 mM L-glutamine) and encapsulated in the HA hydrogels at a density of 20×10^6 cells/mL. Constructs were cultured in chondrogenic media (DMEM-HG, 100 nM dexamethasone, 1% ITS+ supplement, 1% PS, 40 μg/mL L-proline, 1 mM sodium pyruvate, 50 μg/mL ascorbic acid 2-phosphate, 10 ng/mL TGF-β3) at 37 °C and 5% CO_2 for up to 2 weeks. Cell viability was assessed visually using the Live/Dead cytotoxicity kit (Molecular Probes) or quantified using the MTT viability assay (ATCC). For histological analysis, constructs were fixed in 10% formalin for 24 h, embedded in paraffin, and processed using standard histological procedures. The histological sections (7 μm thick) were stained with hematoxylin and eosin (H&E) to observe cell morphology and distributions within the hydrogel and immunohistochemistry for chondroitin sulfate using the Vectastain ABC kit (Vector Laboratories) and the DAB Substrate kit for peroxidase (Vector Laboratories). Sections were predigested in 0.5 mg/mL hyaluronidase for 30 min at 37 °C and incubated in 0.5 N acetic acid for 4 h at 4 °C to swell the samples prior to overnight incubation with primary antibodies at a dilution of 1:100 (mouse monoclonal antichondroitin sulfate, Sigma).

Results and Discussion

Hydrolytically Degradable HA Macromer Synthesis. Our approach involved the inclusion of hydrolytically degradable repeat units of α-hydroxy esters (e.g., lactic acid) between the HA and the polymerizing moiety (e.g., methacrylate). Since poly(lactic acid) is highly versatile in design, hydrolyzable, and approved by the FDA for several biomedical applications[23,24] it was an ideal group to incorporate into the hydrogel. The goal was to design a hydrogel that is tunable with respect to degradation and structure for wide applicability, rather than to meet the specific criteria for one application. The general procedure for the macromer synthesis is outlined in Scheme 1. First, lactic acid was polymerized off the hydroxyl terminal group of HEMA using a ring opening polymerization of lactide monomer in the bulk phase with stannous octoate (tin 2-ethylhexanoate) as a catalyst to form a hydroxyl terminated methacrylated poly(lactic acid) (MeLA-OH). The polymerization proceeds through a coordination-insertion mechanism and the number of lactic acid repeat units is readily controlled through the stoichiometric amount of lactide monomer to HEMA used in the reaction. The ^{1}H NMR spectrum of this polymer (Figure S1) displayed characteristic resonances for the methacrylate protons at δ 6.12 and 5.60 ppm and −CH and $-CH_3$ protons of lactic acid at δ 5.19 and 1.52 ppm, respectively. From the integration ratio of the −CH proton corresponding to the lactic acid units to the methylene protons of the methacrylate group, the number of lactic acid repeat units was estimated (~3 for reported experiments).

Importantly, the number of degradable units can be used to control the hydrolysis rate of hydrogels incorporating lactic acid,[25] with more repeat units leading to more rapid degradation based on the probability of ester cleavage. Also, caprolactone could be substituted for lactide to incorporate a more slowly hydrolyzable group into the hydrogel. For coupling to HA, it

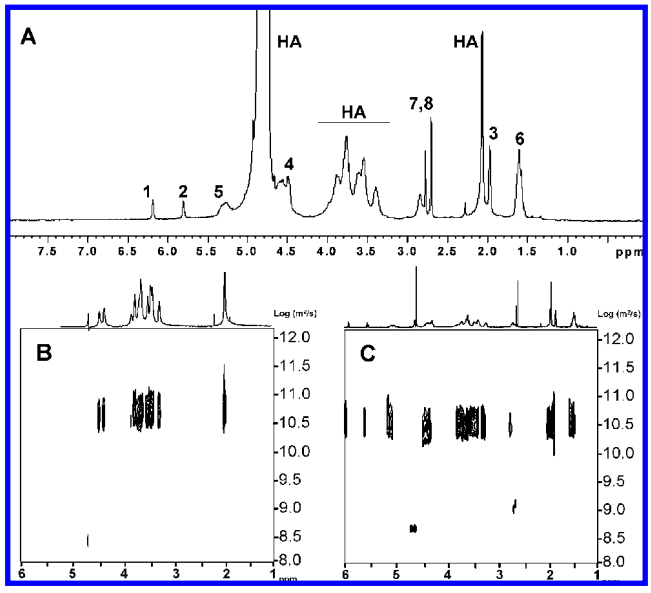


Figure 1. NMR characterization of the synthesized MeLAHA macromer. ^{1}H NMR of the MeLAHA macromer with peaks matching labels of the final structure in Scheme 1 (A) and DOSY spectra of unmodified HA (B) and MeLAHA (C), indicating complete coupling.

was not possible to directly react the free hydroxyl group of MeLA-OH with HA because HA possesses both carboxylic acid and hydroxyl groups that would interfere with the reaction. Thus, the hydroxyl group of lactic acid was converted to a carboxylic acid through an esterification reaction with succinic anhydride in the presence of pyridine and dimethylaminopyridine (DMAP) as an esterification catalyst (Scheme 1) to form a carboxyl terminated methacrylated poly(lactic acid) (MeLA-COOH). The reaction was carried out in anhydrous tetrahydrofuran (THF) because the acid anhydride is subjected to hydrolysis in aqueous medium. The conversion of $-$OH to $-$COOH was confirmed by ^{1}H NMR (Figure S2) with the δ 2.72 ppm resonance corresponding to the $-(CH_2)_2$ group of succinic anhydride observed in the spectrum.

Initial attempts to synthesize the acid chloride derivative of MeLA-COOH by reacting with thionyl chloride to couple to the carboxylic acid groups of HA were unsuccessful due to homopolymerization of the methacrylate groups. Thus, the *N*-hydroxysuccinimidyl ester derivative was formed for coupling to HA through reaction of MeLA-COOH with NHS in the presence of DCC (Scheme 1), where DCC promotes esterification by reacting with the end carboxyl group through nucleophilic substitution. The final product (MeLA-NHS) was obtained after filtering dicyclohexylurea (DCU) as the byproduct and ^{1}H NMR confirmed successful modification (Figure S3).

Because the solubility of the sodium salt of HA (Figure S4) is limited to aqueous solutions and the MeLA-NHS is not water-soluble, HA was converted to its TBA salt (Figure S5) to make it soluble in highly polar organic solvents. After freeze-drying, HA-TBA was dissolved in DMSO and reacted with triethylamine (TEA) and MeLA-NHS for coupling (Scheme 1). The product was converted back to the sodium salt and precipitated in acetone to form MeLAHA. The derivatization reaction was confirmed by ^{1}H NMR analysis (Figure 1A) and exhibited distinct resonances from the $-CH_3$ protons of lactic acid at δ 1.58 ppm and the two protons of the methacrylate at δ 6.18 and 5.80 ppm. The degree of modification ($\sim$10.5%) was determined by the peak areas of the HA backbone and those of the methacrylate groups.

DOSY was used to analyze the purified MeLAHA product (Figure 1B,C) to discriminate between different components of the sample by their chemical shift and diffusion behavior simultaneously. The separation in the diffusion dimension among various peaks is based on the self-diffusion coefficient of different species present in the solution. In DOSY spectrum, molecules with lower molecular weights exhibit higher diffusion coefficients compared to polymers. However, if the same small molecule is attached to the polymer chain, the self-diffusion coefficient will be similar to the polymer. Comparing DOSY spectra in Figure 1B,C, it is clear that peaks due to MeLA (1.0–3.0 ppm and >5.0 ppm) show a similar diffusion coefficient to the HA peaks and confirms the coupling of MeLA to HA. In contrast, physical mixing of MeLA and HA shows significantly higher diffusion coefficient values for MeLA compared to HA (Figure S6). In addition, a slight reduction in the overall diffusion coefficient of MeLAHA was observed after coupling, indicating a higher molecular weight compared to unmodified HA.

Hydrolytically Degradable HA Hydrogel Characterization. With successful synthesis of the MeLAHA macromer, hydrogels were formed and characterized to illustrate the unique properties that are obtained with the new macromer design. The parameters that are readily controlled in this system include the molecular weight of the HA, the type (e.g., lactic acid versus caproic acid) and number (n) of hydrolytically degradable groups, the extent of coupling (percent of HA repeat units modified) of the degradable groups to the HA backbone, and the concentration of macromer used for hydrogel formation. In this study, one macromer (molecular weight $\sim$ 64 kDa, $n \sim$ 3.0, modification $\sim$ 10.5%) was synthesized and polymerized at various concentrations ($\sim$ 1, 2, and 4 wt%) using a photoinitiated polymerization with a growth factor (VEGF) present. These homopolymer hydrogels were placed in PBS, and the release of VEGF and a degradation product (uronic acid) was monitored with time.

Notably, all of the hydrogels completely degraded within 9 days without hyaluronidases present, whereas a control hydrogel formed from MeHA without lactic acid units exhibits minimal degradation in this time period unless enzyme is added. Specifically, the MeHA hydrogels exhibit some uronic acid release within the first 24 h ($\sim$15–20%) and then no further release over the 9 day period. The initial release is attributed to potential sol fraction of the networks and incomplete polymerization at the polymer surface which is exposed to radical-quenching oxygen species. These new hydrogels degrade into HA, lactic acid, and kinetic chains of poly(methacrylic acid). The release of VEGF and degradation products from the MeLAHA hydrogels (Figure 2) was dependent on the macromer concentration during hydrogel formation, with faster degradation and growth factor delivery observed for more loosely cross-linked hydrogels (i.e., lower macromer concentrations).

A burst of VEGF release (>50%) was observed for all hydrogels, but the time for complete degradation and, consequently, VEGF release was dependent on the macromer concentration with the $\sim$1, 2, and 4 wt% hydrogels degrading in $\sim$3, 5, and 9 days, respectively. This is expected because more time is needed for hydrolysis of the greater number of cross-links is needed for complete degradation. One advantage to these hydrogel systems is that there is 100% encapsulation efficiency and release because the VEGF is directly incorporated into the hydrogel network during polymerization. Assessment of the released VEGF activity is beyond the scope of this study, but our laboratory and others have released active growth factors

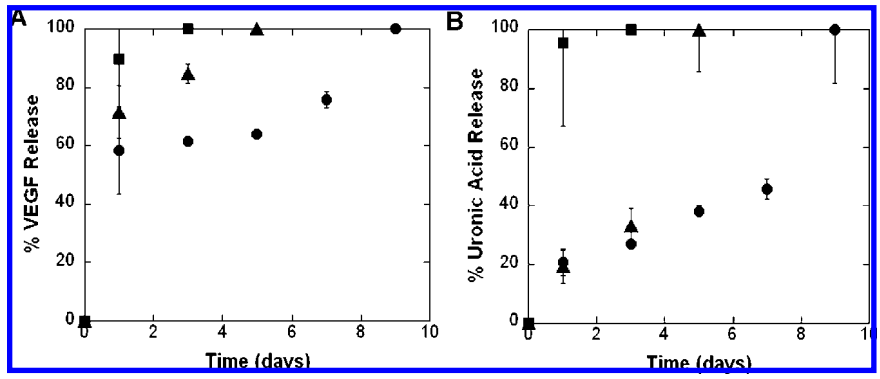


Figure 2. Degradation of hydrogels formed from MeLAHA macromers. VEGF (A) and uronic acid (B) release from pure MeLAHA hydrogels ($n \sim 3.0$, modification $\sim$ 10.5%) at various macromer concentrations [$\sim$1 (■), 2 (▲), and 4 (●) wt%]. Both values are plotted as the cumulative percent release measured using quantitative analyses.

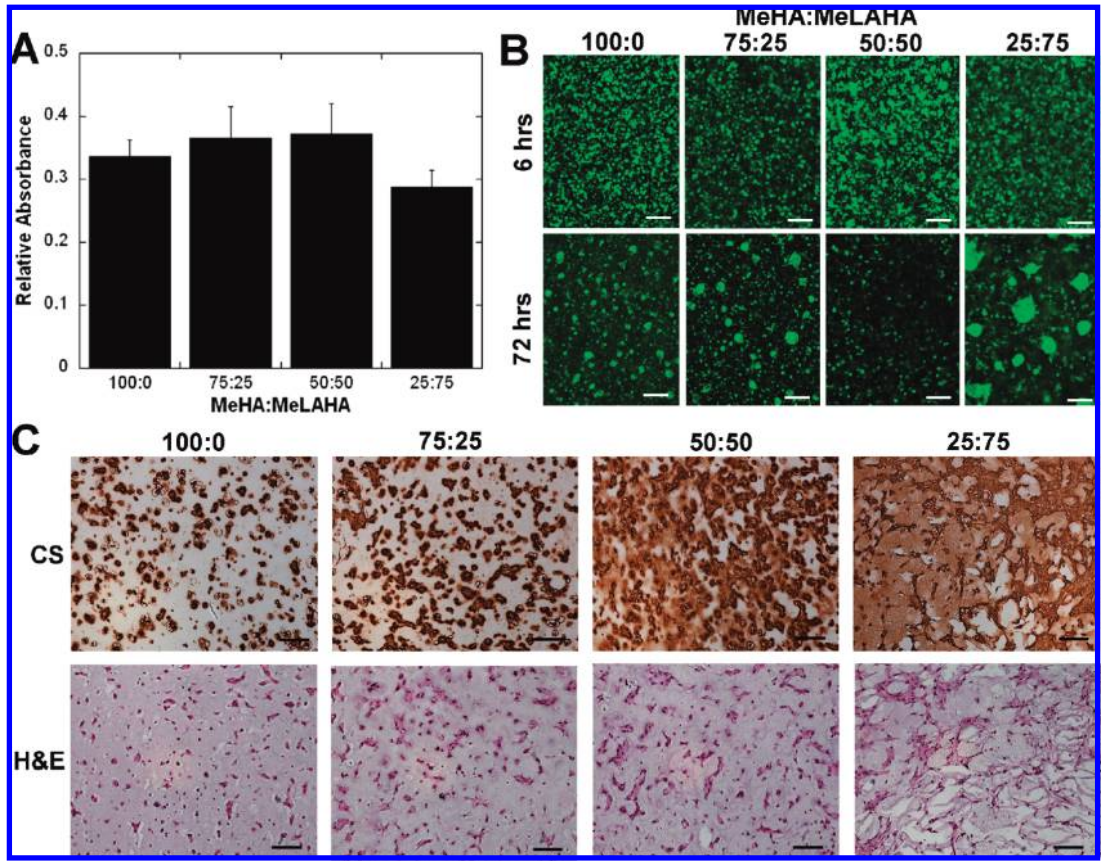


Figure 3. MSC viability and interactions with copolymer HA hydrogels. Mitochondrial activity (MTT assay) after 7 days (A), Live/Dead after 6 and 72 h (B), and histology after 14 days (CS = chondroitin sulfate, H&E = hematoxylin and eosin) of MSCs encapsulated in copolymer HA hydrogels (MeHA/MeLAHA 100:0, 75:25, 50:50, 25:75; scale bar = 200 μm for Live/Dead and 100 μm for histology).

from photocrosslinked and biodegradable hydrogels.[26–28] VEGF was chosen for release due to the importance of HA on vascular differentiation.[17,18,20] Due to the control that is obtained through not only macromer concentration, but other parameters (e.g., type of degradable units), a wide range of release profiles is possible.

Cellular Interactions with Hydrolytically Degradable HA Hydrogels. For tissue engineering applications, the ability to entrap cells and to control cellular behaviors and tissue formation is essential. In this work, MSCs were chosen due to their ability to differentiate into various phenotypes and their clinical potential.[29,30] Because the synthesized MeLAHA macromer degrades fairly quickly, it was copolymerized with MeHA (molecular weight $\sim$ 64 kDa, modification $\sim$ 35%) at various ratios (100:0, 75:25, 50:50, 25:75, 0:100 MeHA/MeLAHA) and a concentration of $\sim$2 wt% to obtain a wide range of networks exhibiting differential temporal structures. The MSCs remained viable (>95%) after encapsulation in all hydrogels, as evident by the mitochondrial activity after 7 days (Figure 3A) and Live/Dead staining after 6 h and 3 days (Figure 3B). The 0:100 MeHA/MeLAHA degraded in less than 3 days and could not be analyzed further. The mitochondrial activity is similar between the groups after 7 days, but is slightly lower in the 25:75 MeHA/MeLAHA hydrogels, which may indicate differences in MSC proliferation. Importantly, the cells were homogenously viable in the constructs, yet the cellular morphology changed depending on the copolymer concentrations, with the greatest amount of hydrolytically degradable macromer leading to more cell clustering by the 3 day time point (Figure 3B).

When the distribution of tissue produced by encapsulated MSCs was assessed, differences were also observed (Figure 3C) after 2 weeks of culture in chondrogenic media. In both chondroitin sulfate (CS) and hematoxylin and eosin (H&E) staining, greater molecule distribution and macroporosity were observed with increasing inclusion of the hydrolytically degradable macromer. Specifically, a gradient of CS (an important extracellular matrix molecule in cartilage) distribution corresponded with the amount of MeLAHA incorporated. This is an important finding in that more uniform tissues can be produced by altering degradation profiles with addition of this newly synthesized macromer. Others have utilized differential degradation profiles in purely synthetic hydrogels to enhance uniformity in matrix distribution.[31,32]

HA hydrogels have been modified by others to manipulate degradation behavior. For instance, methacrylated HA has been copolymerized with a methacrylated version of aspartamide to form dynamic gels for drug delivery.[33] However, this work involves the addition of a potentially toxic reagent to control the hydrogel properties, the synthesized hydrogels did not degrade completely in the absence of enzymes (incomplete susceptibility to hydrolysis), and no efforts were made to encapsulate cells in the hydrogels. Thus, the properties attained with this novel hydrogel presented here are advantageous for certain applications.

Although cartilage was used here as a model system, this approach has wide implications in the engineering of many tissues, specifically because degradation does not rely on potential tissue-specific enzymes. Importantly, others have found that the hydrogel chemistry and properties (e.g., mechanics and degradation) may play a role in the differentiation of stem cells.[34,35] These parameters of the stem cell microenvironment must be controlled in the design of tissue engineering systems and may also be exploited for controlled differentiation, and thus it is important to have precise control over the material properties. This is possible with this reported material since degradation occurs via multiple mechanisms and can be altered to meet specific criteria.

Conclusions

In summary, we present the synthesis of a novel macromer that forms HA hydrogels that are hydrolytically degradable and have enhanced control over previously synthesized versions. The synthetic scheme utilized several steps toward the coupling of a reactive methacrylate group polymerized with hydrolytically degradable repeat units to HA to form a macromer that can be photopolymerized into hydrogels for the encapsulation of growth factors or cells. The degradation of these hydrogels and subsequent growth factor release can be controlled by numerous factors, including the hydrogel cross-linking density. Finally, these hydrogels and copolymer hydrogels (with purely enzymatically degradable HA macromers) supported the encapsulation of viable cells and the cellular morphology and the distribution of extracellular matrix molecules was dependent on the amount of hydrolytically degradable HA macromer. These results indicate advanced engineering control over HA hydrogels toward a variety of applications.

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Supporting Information Available. A detailed description of the MeLAHA macromer synthesis, including synthetic details, reaction yields, and NMR spectra for each reaction step. This material is available free of charge via the Internet at http://pubs.acs.org.

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