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The Effect of Desulfation of Chondroitin Sulfate on Interactions with Positively Charged Growth Factors and Upregulation of Cartilaginous Markers in Encapsulated MSCs

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Abstract

Sulfated glycosaminoglycans (GAGs) are known to interact electrostatically with positively charged growth factors to modulate signaling. Therefore, regulating the degree of sulfation of GAGs may be a promising approach to tailor biomaterial carriers for controlled growth factor delivery and release. For this study, chondroitin sulfate (CS) was first desulfated to form chondroitin, and resulting crosslinked CS and chondroitin hydrogels were examined *in vitro* for release of positively charged model protein (histone) and for their effect on cartilaginous differentiation of encapsulated human mesenchymal stem cells (MSCs). Desulfation significantly increased the release of histone from chondroitin hydrogels (30.6 ± 2.3 μ g released over 8 days, compared to natively sulfated CS with 20.2 ± 0.8 μ g), suggesting that sulfation alone plays a significant role in modulating protein interactions with GAG hydrogels. MSCs in chondroitin hydrogels significantly upregulated gene expression of collagen II and aggrecan by day 21 in chondrogenic medium (115 ± 100 and 23.1 ± 7.9 fold upregulation of collagen II and aggrecan, respectively), compared to CS and PEG-based swelling controls, indicating that desulfation may actually enhance the response of MSCs to soluble chondrogenic cues, such as TGF- β 1. Thus, desulfated chondroitin materials present a promising biomaterial tool to further investigate electrostatic GAG/growth factor interactions, especially for repair of cartilaginous tissues.

Keywords

chondroitin sulfate; glycosaminoglycan; growth factors; mesenchymal stem cell; cartilage tissue engineering

1. Introduction

Sulfated glycosaminoglycans (GAGs), including heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate, are highly negatively charged unbranched

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polysaccharides that occur naturally throughout the body, largely associated with proteoglycans [1]. Sulfated GAGs are known to play an important role in maintaining structural integrity and extracellular matrix (ECM) organization within connective tissues; however, the complexity and diversity of these GAGs suggest that they also play critical roles in modulating macromolecular binding and signaling in biological processes [2]. GAG proteoglycans have been found to alter cellular signaling, both as ECM components and on the cell surface, largely through electrostatic interactions with charged proteins [2]. Heparin and heparan sulfate especially are known to play important roles in sequestration and signaling of positively charged growth factors *in vivo* [3, 4]. These growth factor interactions regulate critical signals in development and normal mammalian physiology, including growth factor/receptor binding, restricted diffusion of cytokines, and developmental tissue patterning [5, 6].

The ability of sulfated GAGs to sequester growth factors has been exploited, primarily with heparin, as drug delivery vehicles for controlled delivery and release [7-9]; therefore, further modulating protein interactions with GAGs remains a critical goal in order to provide greater control of growth factor-carrier affinity for a variety of applications, including controlled release for tissue engineering. Because the sulfate groups in GAGs contribute to their highly negative fixed charge density, facilitating electrostatic interactions with basic amino acid residues in positively charged growth factors [2], growth factor interactions may potentially be controlled by altering the degree of sulfation in GAG materials for precise control over their release kinetics without significant changes in the total material composition.

Compared with heparin, growth factor interactions with other GAGs have not yet been as well characterized; however, chondroitin sulfate (CS)-containing proteoglycans appear to play important roles in growth factor signaling *in vivo*, particularly during chondrogenesis [10-12], suggesting that growth factor binding abilities are not restricted to heparin. Previous work has shown that oversulfation of CS enhances its binding affinity in a sulfation-dependent manner *in vitro*, including to the chondrogenic growth factor transforming growth factor- β 1 (TGF- β 1), supporting that the degree of sulfation plays a critical role in regulating CS interactions with growth factors [13, 14]; however, desulfation of similar CS materials has not yet been examined for its effect on growth factor interactions. Therefore, in this work, CS was first chemically desulfated, and the resulting chondroitin materials were characterized for sulfation pattern and total charge. CS and chondroitin materials were then methacrylated to facilitate crosslinking to form hydrogels, and the ability of CS and chondroitin hydrogels to interact with positively charged proteins was examined.

As a major component of cartilaginous tissues, CS also appears to play a particularly important role in directing stem cell differentiation down a chondrogenic lineage *in vitro*. Previous work has shown that culture in CS-containing poly(ethylene glycol) (PEG) hydrogels upregulated expression and production of cartilaginous ECM by encapsulated goat and mouse mesenchymal stem cells (MSCs) [15, 16]; however, relatively few studies have specifically investigated the effect of CS on chondrogenic differentiation of human MSCs [17, 18], and to our knowledge, no studies to date have examined the role of desulfated chondroitin materials in this context. Comparison of CS materials to a desulfated form of chondroitin presents a highly controlled model system to examine the roles of sulfation and growth factor interactions in localized cell response without major structural modification of the GAG-based microenvironment. Therefore, in these studies, human MSCs were encapsulated in PEG-based hydrogels containing 50% w/w CS or 50% w/w chondroitin and cultured in medium either with or without TGF- β 1 over the course of 3 weeks *in vitro*. Cell viability and total DNA content were monitored over time, and gene expression and ECM production of encapsulated MSCs were determined with quantitative

reverse transcription polymerase chain reaction (RT-PCR) and immunostaining, respectively, as measures of chondrogenic differentiation.

2. Materials and Methods

2.1. Desulfation of Chondroitin Sulfate and Characterization of Desulfated Chondroitin

2.1.1. Desulfation of Chondroitin Sulfate—Chondroitin sulfate was desulfated using an acidic methanol treatment for up to 7 days per established protocols [19]. Chondroitin sulfate C ($M_n = 16,300$ Da; Wako Chemicals USA, Richmond, VA) was stirred at 5.0 mg/mL in methanol (VWR, Radnor, PA) containing 0.5% v/v acetyl chloride (Acros Organics, Geel, Belgium). CS was centrifuged and acidic methanol was replaced either on days 1, 2, and 3 for a 3-day reaction or on days 1, 3, and 7 for a 7-day reaction to produce a methyl ester of chondroitin (Figure 1a). The product was then dissolved in 20 mL distilled, deionized water (ddH₂O) per gram of starting CS before precipitation in an excess of ethanol. The methyl ester of chondroitin was washed in ethanol and ethyl ether (Fisher, Waltham, MA), vacuum dried at <5 mmHg, and stored at 4°C.

Methyl ester of chondroitin was demethylated at 25 mg/mL in 0.1 M potassium hydroxide (KOH, Fisher) for 24 hours to produce chondroitin (Figure 1a). The chondroitin product was then neutralized in 4 mL 100 mg/mL potassium acetate (Fisher) in 10% v/v acetic acid (VWR) per gram of starting product, and precipitated in an excess of ethanol. Chondroitin was washed in ethanol and ethyl ether, vacuum dried, and stored at 4°C until use.

2.1.2. Dimethylmethylene Blue Assay—Removal of sulfate groups in chondroitin was confirmed by dimethylmethylene blue (DMMB) assay for sulfated GAGs. Standard curves from 0 to 50 µg/mL chondroitin or chondroitin sulfate were assayed by DMMB according to established protocols [20], and absorbance was measured at 520 nm in a plate reader (SpectraMax M2e; Molecular Devices, Sunnyvale, CA). The slopes of the chondroitin standard curves were compared to the CS standard curve to determine relative desulfation of the chondroitin product.

2.1.3. Fourier Transform Infrared Spectroscopy—To further verify the removal of sulfate groups in chondroitin, CS and chondroitin materials were analyzed by Fourier transform infrared (FTIR) spectroscopy. A 3 mg/mL solution of CS or desulfated chondroitin in deuterated water (D₂O; Sigma-Aldrich, St. Louis, MO) was spin coated onto a silicon crystal, and FTIR was measured using a Bruker Vertex 70 ATR-FTIR spectrometer (Billerica, MA) with atmospheric compensation.

2.1.4. Strong Anion Exchange High Performance Liquid Chromatography—Samples were analyzed by strong anion exchange high performance liquid chromatography (SAX-HPLC) by the University of Georgia Complex Carbohydrate Research Center to determine the disaccharide composition and average charge density of the CS and chondroitin materials. A 1 mg/mL GAG solution of either CS or chondroitin was digested into disaccharides by 0.1 mU/mL of chondroitinase ABC (Sigma-Aldrich) at 37°C for 24 hours. The chondroitinase enzyme was then inactivated by heating to 100°C for 2 minutes, and the sample was centrifuged prior to HPLC analysis.

SAX-HPLC was carried out on an Agilent 1200 system (Santa Clara, CA) using a Waters Spherisorb analytical column (4.6×250 mm, 5 µm particle size; Milford, MA) at 25°C, using an injection volume of 10 µL and a flow rate of 1.0 mL/min. The 2.5 mM sodium phosphate (Na₃PO₄) solvent at pH 3.5 was gradually transitioned up to a 1.2 M NaCl concentration over the course of 55 minutes. Disaccharide detection was performed by post-column derivatization, as a 1:1 mixture of 0.25 M NaOH and 1% 2-cyanoacetamide was added to

the eluent from the column from a binary HPLC pump at 0.5 mL/min, heated to 120°C in a 10-m reaction coil, followed by cooling in a 50-cm cooling coil, and directed into a Shimadzu fluorescence detector (excitation: 346 nm, emission: 410 nm; Kyoto, Japan). Based on these results, a predictive model was used to calculate average charge, based on known disaccharide structures containing negatively charged sulfate and carboxylate groups.

2.1.5. Size Exclusion High Performance Liquid Chromatography—CS and chondroitin samples were analyzed by size exclusion high performance liquid chromatography (SEC-HPLC) by the University of Georgia CCRC to determine the average molecular weight of the CS and chondroitin chains. Solutions of CS or chondroitin were prepared at 2 mg/mL in 50 mM sodium sulfate (Na_2SO_4) buffer, pH 5.0, and partially depolymerized heparin fractions were used as molecular weight standards. Separations were carried out using a TSKGel G3000SWXL column (Tosoh Bioscience, Stuggart, Germany, 7.8 mm ID $\times$ 30 cm) and a TSKGel G2000SWXL column (7.8 mm ID $\times$ 30 cm), connected in series, on an Agilent 1200 LC instrument using refractive index detection with an injection volume of 50 μL and a flow rate of 0.5 mL/min.

2.2. Synthesis and Characterization of Crosslinkable Hydrogel Materials

2.2.1. Methacrylation of Chondroitin Sulfate and Chondroitin—CS and chondroitin were methacrylated with glycidyl methacrylate per established protocols [21]. CS or chondroitin was dissolved at 1% w/v in a 50:50 mixture of acetone (VWR) and ddH_2O and allowed to stir at room temperature overnight. A 20-fold molar excess of triethylamine (TEA, Sigma-Aldrich) per CS or chondroitin disaccharide was added to the solution, and a 20-fold molar excess of glycidyl methacrylate (GMA, Sigma-Aldrich) per disaccharide was then added dropwise to the reaction mixture. The reaction was allowed to stir at room temperature for 24 hours to produce CS-methacrylate (CS-MA) (Figure 2a) and chondroitin-methacrylate (Ch-MA). The resulting products were dialyzed first in 50:50 acetone:water for 24 hours (1,000 Da MWCO), and then in distilled water (dH_2O) for 2 days to remove unreacted reagents. The methacrylated products were lyophilized (Labconco, Kansas City, MO) for 4 days to produce a dry product, and stored at -20°C until use. Methacrylation of CS and chondroitin materials was verified by proton nuclear magnetic resonance (^1H NMR) in D_2O to determine the degree of conjugation of the GMA groups to the CS and chondroitin chains.

2.2.2. Synthesis of Crosslinkable PEG-Based Materials—For MSC differentiation experiments, poly(ethylene glycol)-diacrylate (PEG-DA) and oligo(poly(ethylene glycol) fumarate) 10K (OPF) polymers were synthesized according to established protocols [22, 23]. The molecular weight distribution of the PEG-DA and OPF products were characterized by gel permeation chromatography (GPC) in chloroform (Fisher) as previously reported [24].

2.2.3. Formation of GAG Hydrogels with Varying Sulfation—To demonstrate that hydrogels could be fabricated with a constant GAG content and varying degrees of sulfation, CS-MA and Ch-MA were mixed in varying mass ratios including 100% w/w Ch-MA (0% CS-MA), 1% w/w CS-MA/99% w/w Ch-MA, 10% w/w CS-MA/90% w/w Ch-MA, 50% w/w CS-MA/50% w/w Ch-MA, 75% w/w CS-MA/25% w/w Ch-MA, and 100% w/w CS-MA (0% Ch-MA). 30 μL of macromer solution at an initial water content of 90% w/w were then crosslinked in 6 mm diameter, 1 mm deep cylindrical molds with 0.018 M ammonium persulfate (APS, Sigma-Aldrich) and tetramethylethylenediamine (TEMED, Sigma-Aldrich) thermal initiator system for 10 minutes at 37°C . After 5 days of swelling in phosphate-buffered saline (PBS) to demonstrate the retention of GAG within the crosslinked networks, hydrogels were stained overnight in DMMB solution ($n=2$) and imaged visually.

2.2.4. Degradation of Modified Chondroitin Sulfate and Chondroitin Materials

—To determine if the ability of chondroitinase enzyme to degrade CS was affected by either desulfation, methacrylation, or crosslinking, the degradation of modified CS-MA and Ch-MA was determined both in solution and in crosslinked hydrogels. Soluble chondroitinase activity was assayed per established protocols [25]. 2 mg/mL CS-MA and Ch-MA solutions were incubated with 0.06 U/mL chondroitinase ABC at pH 8.0. Accumulation of the $\Delta^{4,5}$ -unsaturated disaccharide degradation product at 37°C was monitored by measuring the increase in absorbance at 232 nm UV light in a UV-transparent 96-well assay plate (Corning Incorporated, Corning, NY) by a plate reader over the course of 1 hour, compared to CS-MA and Ch-MA blanks incubated without enzyme (n=3).

To determine the ability of crosslinked CS-MA and Ch-MA hydrogels to degrade in the presence of chondroitinase enzyme, 100% CS-MA or 100% Ch-MA materials were crosslinked with 0.018 M APS/TEMED as described in Section 2.2.3. and swelled overnight in PBS. CS-MA and Ch-MA hydrogels were then transferred into 0.125 U/mL chondroitinase ABC and incubated at 37°C. Complete degradation was determined when the bulk hydrogel was no longer visible in solution (n=4).

2.2.5. Swelling of CS and Chondroitin Hydrogels—Macromer solutions containing 100% Ch-MA, 10% CS-MA/90% Ch-MA, 50% CS-MA/50% Ch-MA, or 100% CS-MA were crosslinked with 0.018 M APS/TEMED as described in Section 2.2.3. and swelled overnight in PBS. After 1 day swelling in PBS, the wet weight of crosslinked hydrogels was recorded, and following lyophilization overnight, dry weight was recorded. Swelling ratio was calculated as wet weight/dry weight (two independent experiments of n=5).

2.2.6. Release of Positively Charged Histone by CS and Chondroitin Hydrogels—To investigate the role of sulfation in the electrostatic complexation and release of histone as a positively charged model protein, macromer solutions containing 100% Ch-MA, 10% CS-MA/90% Ch-MA, 50% CS-MA/50% Ch-MA, or 100% CS-MA were loaded with 40 μ g histone (~14.0 kDa) per 30 μ L hydrogel, and crosslinked with 0.018 M APS/TEMED as described in Section 2.2.3. Loading correlated to a ~1:3,000 histone:disaccharide molar ratio in GAG hydrogels. Hydrogels were incubated at 37°C in 500 μ L PBS. At 3 and 12 hours, and after 2, 4, and 8 days, the supernatant was sampled and analyzed for total protein content by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL), and cumulative release was calculated for each hydrogel over 8 days (n=5).

2.3. Chondrogenic Differentiation of Human MSCs Encapsulated in PEG Hydrogels Containing CS-MA and Ch-MA

2.3.1. Encapsulation of MSCs in Hydrogels—Human MSCs were obtained from the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White (Temple, TX) at passage 1. Cells were seeded at 50 cells/cm² following recommended protocols, in growth medium containing α -MEM (Mediatech, Manassas, VA) with 16.3% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 1% antibiotic/antimycotic (Mediatech), and 2 mM L-glutamine (Mediatech). Following expansion, cells were frozen at passage 2 in liquid nitrogen until further use. For these studies, cells from three separate donors were thawed, expanded separately, and combined prior to encapsulation at passage 3.

As a comparison to previous studies, in which MSCs were encapsulated in PEG-based hydrogels containing CS [15, 16, 18], in the following cell-based experiments PEG-based materials PEG-DA ($M_n = 3,760 \pm 50$ Da, $PI = 1.1 \pm 0.02$) and OPF ($M_n = 28,100 \pm 760$ Da, $PI = 5.2 \pm 0.4$) were incorporated into hydrogels at mass ratios of 25% each with either 50% CS-

MA or 50% Ch-MA as depicted in Table I. Fold swelling of the PEG-based hydrogels was characterized as described in Section 2.2.5, and a 60% PEG-DA/40% OPF mixture that possessed similar swelling properties to 50% Ch-MA formulations was used as a PEG-only swelling control.

50% CS-MA, 50% Ch-MA, and PEG swelling control polymer solutions were filter sterilized through a 0.2 μm pore filter, and dispersed human MSCs were incorporated at a cell concentration of 10×10^6 cells/mL and sterilely crosslinked in hydrogels with 0.018 M APS/TEMED as described in Section 2.2.3. Hydrogels were cultured for 3 weeks at 37°C and 5% CO_2 in basal medium composed of high glucose DMEM (Mediatech) containing 1% ITS+ culture supplement (Becton, Dickinson, Franklin Lakes, NJ), 1% nonessential amino acids (NEAA, Mediatech), 50 $\mu\text{g/mL}$ ascorbate-2-phosphate (Sigma-Aldrich), and 1% antibiotic/antimycotic, or in chondrogenic medium supplemented with 10 ng/mL TGF- β 1 (Peprotech, Rocky Hill, NJ) and 100 nM dexamethasone (Sigma-Aldrich). Culture medium was replaced every 2 days throughout the course of the study.

2.3.2. LIVE/DEAD Staining for Viability—Viability of human MSCs encapsulated in 50% CS-MA and 50% Ch-MA hydrogels was observed by LIVE/DEAD staining over 3 weeks. On days 1, 7, 14, and 21, hydrogels were stained for 60 minutes in LIVE/DEAD stain containing 1 μM calcein and ethidium homodimer-1 (Invitrogen). Viability within all hydrogel formulations was imaged via confocal microscopy (viable cells = green, ex/em: 494/517 nm; nonviable cells = red, ex/em: 528/617 nm; Carl Zeiss LSM 510, Oberkochen, Germany). Images were captured every 10 μm through the entire depth of the hydrogel from three separate regions in each sample (n=4).

2.3.3. PicoGreen DNA Assay—Hydrogels were analyzed by PicoGreen assay for DNA content on days 1, 7, 14, and 21. After rinsing in PBS, samples were massed, and wet mass was recorded. Hydrogels were then homogenized with pestle grinders and mixed with 500 μL of distilled water before being frozen at -80°C until analysis. Cells were lysed through a series of freeze/thaw cycles and sonication. PicoGreen assay (Invitrogen) was used to evaluate the total DNA content in each sample, according to established protocols [26]. Fluorescence was read at excitation 485 nm, emission 525 nm by a plate reader, and DNA content was determined using a standard curve of DNA. Within each hydrogel formulation, DNA content of each gel was normalized to wet mass to correct for small differences in gel size (n=4).

2.3.4. Reverse Transcription Polymerase Chain Reaction—Gene expression of encapsulated MSCs was analyzed after 1, 7, 14, and 21 days by reverse transcription polymerase chain reaction. RNA was extracted from samples using a QIAshredder tissue homogenizer and RNeasy kit with DNase I digestion (Qiagen, Hilden, Germany). Reverse transcription was performed using SuperScript III Reverse Transcriptase (Invitrogen) with Oligo(dT)₁₅ primers (Promega, Madison, WI) and nucleotides (Promega). Custom designed primers (Invitrogen) specific to human mRNA for collagen II, aggrecan, and SOX9 (chondrocytic markers), collagen X (hypertrophic chondrocyte marker), collagen I (fibroblastic marker), osteocalcin (osteoblastic marker), myoD (myofibroblastic marker), and peroxisome proliferator-activated receptor γ 2 (PPAR- γ 2; adipocytic marker) are shown in Table II. Quantitative PCR amplification for each gene target was performed on a StepOnePlus System (Applied Biosystems, Carlsbad, CA) with SYBR Green master mix (Applied Biosystems). To determine fold regulation over PEG control hydrogels on day 1, the raw fluorescence data was processed using LinRegPCR (v12.11; <http://www.hartfaalcentrum.nl>) [27] with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control (n=6).

2.3.5. Histological Staining—ECM production by encapsulated MSCs was determined by immunostaining on days 1, 7, 14, and 21. Samples were infiltrated with a solution of sucrose (EMD, Darmstadt, Germany) and OCT (VWR) in PBS under vacuum (−25 inHg), embedded in 1:2 20% sucrose:OCT solution by gentle freezing in liquid nitrogen, and stored at −80°C. Infiltrated hydrogels were cryosectioned at 20 μ m thickness (Thermo Scientific, Cryostar NX70).

Prior to staining, frozen sections were fixed in acetone. For aggrecan and collagen X staining, samples were deglycosylated with 30 μ l of 0.75 U/ml chondroitinase ABC for 1.5 hours. Samples were blocked with Image-iT FX signal enhancer (Invitrogen) for 30 minutes for use with IgG primary antibodies or with a solution of 2% normal goat serum for 20 minutes for use with anti-collagen X IgM primary antibodies. For primary antibody binding, sections were incubated overnight at 4°C in monoclonal mouse anti-human collagen I, collagen II, aggrecan (IgG1, Abcam), or collagen X (IgM, Sigma). Sections were then incubated with highly cross-adsorbed Alexa Fluor 488-conjugated goat polyclonal anti-mouse IgG or IgM (Molecular Probes, Carlsbad, CA), and counterstained with 0.1 μ g/mL 4',6-diamidino-2-phenylindole (DAPI, Anaspec, Fremont, CA). Negative controls were stained as described, but using a monoclonal mouse IgG1 isotype control or monoclonal mouse IgM isotype control (Abcam) with no known reactivity with human antigens as the primary antibody. Histological sections were imaged using an epifluorescence microscope with a 20X magnification objective (n=2; Nikon Eclipse 80i, Tokyo, Japan).

2.4. Pull-Down (Depletion) of TGF- β 1 from Solution by CS-MA and Ch-MA Hydrogels

To investigate the role of sulfation in TGF- β 1 sequestration by GAG-based hydrogels, TGF- β 1 pull-down (or depletion) from solution by PEG-based hydrogels containing varying amounts of CS-MA or desulfated Ch-MA was investigated. Acellular 50% CS-MA, 50% Ch-MA and 60% PEG-DA/40% OPF hydrogels were fabricated as described in Section 2.3.1., according to the formulations in Table I. After unconstrained swelling in PBS overnight, hydrogels were incubated in 1.0 mL solution of 2.0 ng/mL TGF- β 1 in 1% bovine serum albumin (BSA) in PBS for 24 hours at 37°C with gentle shaking. To inhibit electrostatic binding with hydrogels, pull-down was also measured with an additional 0.5 M NaCl or 10 mg/mL soluble CS in the TGF- β 1 solution. The TGF- β :disaccharide ratio in the solutions correlated to a ~1:50,000,000 molar ratio in 50% GAG hydrogels. After 24 hours, the supernatant was collected and frozen at −20°C until analysis. TGF- β 1 pull-down by the hydrogels was determined by assaying the remaining TGF- β 1 in solution by ELISA (n=5).

2.5. Statistical Analysis

All values were reported as mean $\pm$ standard deviation. For statistical analysis, PCR amplification data for each gene were first transformed using a Box-Cox transformation to obtain a normal distribution for analysis [28]. A one- or two-factor analysis of variance (ANOVA) was used to determine statistical significance of groups, and Tukey's *post hoc* multiple comparison test with significance set at p 0.05 indicated significance between individual samples. For all one-factor ANOVAs (swelling and histone release), the factor was hydrogel type. For DNA and gene expression analysis, the factors were hydrogel type and time. For pull-down experiments, the two factors were hydrogel type and buffer composition. Statistical analysis was carried out using Minitab (v15.1, State College, PA).

3. Results

3.1. Desulfation of CS by Acidic Methanol Treatment

DMMB assay was used to measure sulfation level of the chondroitin products after acidic methanol treatment. CS only experienced removal of 80% of the sulfates after 3 days

(Figure 1b); however, extension of the acidic methanol treatment time from 3 to 7 days resulted in nearly complete desulfation of CS, as indicated by zero slope in the desulfated chondroitin standard curve by DMMB assay. Therefore, the chondroitin product after 7 days of desulfation in acidic methanol was used in all future experiments, compared to unmodified CS. As further confirmation of desulfation, FTIR spectroscopy was used to examine the bonds present in CS and chondroitin. FTIR spectroscopy verified the disappearance of sulfate peaks at $1100\text{--}1250\text{ cm}^{-1}$ (Figure 1c, black box), while the remaining bonds in CS appeared to remain unchanged.

SAX-HPLC analysis was performed to determine the disaccharide composition of CS and desulfated chondroitin. From disaccharide elution patterns, it was determined that CS disaccharides were approximately 57.8% 6-sulfated and 26.8% 4-sulfated, as well as 1.3% nonsulfated (Table III). The desulfated chondroitin product, however, was 98.5% nonsulfated and only 1.5% 6-sulfated. The observed shift in chondroitin disaccharide composition to a primarily nonsulfated form also indicated a reduction in negative charge density in chondroitin, compared to CS. Based on these results, a predictive model calculated CS to have an average charge of -2.3 per disaccharide, while chondroitin had an average charge of -1.02 , indicating that desulfation resulted in a decrease in negative charge density of CS by over two-fold.

SEC-HPLC analysis was also performed to determine the average molecular weight of the full CS and chondroitin chains. It was determined that CS had a weight averaged molecular mass (M_w) of 17,880 Da and a number averaged molecular mass (M_n) of 16,300 Da ($PI=1.1$). Chondroitin, however, was notably smaller with an M_w of 6,310 Da and an M_n of 5,230 Da ($PI=1.2$). This represented a 67.9% decrease in molecular weight (M_n) from CS, while sulfates only accounted for 17.1% of mass based on disaccharide composition.

3.2. Crosslinked CS-MA/Ch-MA Hydrogels with Varying Degrees of Sulfation

^1H NMR spectra indicated that GMA was successfully conjugated to CS. Vinyl peaks were visible at 5.6 and 6.0 ppm, confirming the methacrylation of CS and chondroitin by GMA to form CS-MA and Ch-MA, respectively (data not shown). Peak integration also indicated that on average one GMA molecule was conjugated per every 4.3 disaccharides in CS-MA and every 3.7 disaccharides in Ch-MA. Soluble methacrylated CS and chondroitin degraded in the presence of chondroitinase ABC with an increase in absorbance of 232 nm UV light over time, indicating an increase of $\Delta^{4,5}$ -unsaturated disaccharide degradation products (data not shown). Complete degradation of CS-MA and Ch-MA occurred within ~30 minutes in solution. Additionally, crosslinked CS-MA and Ch-MA hydrogels completely degraded within 4 hours in chondroitinase enzyme (data not shown).

GAG-based hydrogels were fabricated containing varying ratios of CS-MA/Ch-MA as the fraction of CS-MA was varied with the remainder balanced with nonsulfated Ch-MA. A pink/purple color indicated the presence of sulfated GAG in a concentration-dependent manner. Staining with DMMB indicated that CS-containing hydrogels stained positively for sulfation after 5 days of swelling in PBS, and increasing degrees of staining were apparent in hydrogels as sulfation increased up to 100% CS-MA (Figure 2b).

100% CS-MA and 100% Ch-MA hydrogels possessed similar fold swelling ratios of 26.0 ± 1.4 and 25.5 ± 1.7 fold, respectively (Figure 2c). CS-MA/Ch-MA mixtures of 10% CS-MA/90% Ch-MA and 50% CS-MA/50% Ch-MA hydrogels, however, swelled slightly less than 100% CS-MA and Ch-MA hydrogels with 22.0 ± 2.2 and 21.7 ± 0.9 fold swelling ratios, respectively.

3.3. Release of Histone Protein from CS-MA/Ch-MA Hydrogels

Histone release studies from CS-MA and Ch-MA hydrogels observed increased release of positively charged histone protein correlating with decreased sulfation. Hydrogels containing 100% CS-MA (0% Ch-MA) displayed significantly smaller burst release of histone after 3 hours of swelling with 15.6 ± 0.7 μg histone released (Figure 2d). 50% CS-MA/50% Ch-MA hydrogels demonstrated significantly greater burst release than 100% CS-MA with 19.7 ± 2.0 μg histone released. Additionally, 10% CS-MA/90% Ch-MA and nonsulfated 100% Ch-MA (0% CS-MA) hydrogels exhibited the greatest burst release after 3 hours of swelling with 24.5 ± 2.2 and 22.4 ± 2.0 μg histone, respectively.

After 8 days of release, hydrogels containing 100% CS-MA displayed the least release of histone with 20.2 ± 0.8 μg histone cumulatively released (Figure 2d, $50.4 \pm 1.9\%$ release). 50% CS-MA hydrogels demonstrated significantly greater release than 100% CS-MA with 24.9 ± 2.6 μg ($62.2 \pm 6.4\%$) cumulative histone release after 8 days. Additionally, 10% CS-MA and 100% Ch-MA hydrogels, which were the least sulfated, exhibited the greatest cumulative release of 32.5 ± 3.9 μg ($81.2 \pm 9.8\%$) and 30.6 ± 2.3 μg ($76.4 \pm 5.8\%$) histone, respectively. Even when only considering the additional release following the initial burst release measured at 3 hours of swelling, the least sulfated 10% CS-MA and 100% Ch-MA hydrogels (7.9 ± 2.0 μg and 8.1 ± 0.7 μg additional release, respectively) experienced significantly greater release of histone between 3 hours and 8 days than the more sulfated 100% CS-MA and 50% CS-MA compositions (4.5 ± 0.7 μg and 5.1 ± 0.6 μg , respectively).

3.4. Viability of Encapsulated MSCs

For comparison to previous studies in PEG-based hydrogels, human MSCs were encapsulated in 25% PEG-DA/25% OPF/50% GAG hydrogels (50% CS-MA or 50% Ch-MA). PEG hydrogels containing 50% CS-MA possessed a fold swelling ratio of 25.2 ± 1.4 , while PEG hydrogels containing 50% Ch-MA swelled significantly less than 50% CS-MA with 16.9 ± 2.1 fold swelling (Table I). 60% PEG-DA/40% OPF hydrogels (17.1 ± 0.5 fold swelling) that swelled similarly to 50% Ch-MA hydrogels were therefore fabricated as a PEG-based swelling control for comparison to 50% Ch-MA.

LIVE/DEAD staining of human MSCs in 50% CS-MA and 50% Ch-MA hydrogels indicated that visible MSCs remained mostly viable over 3 weeks of culture (Figure 3a). Cells remained dispersed evenly throughout the hydrogel scaffolds with a spherical shape, and no cell aggregation or spreading was observed. Total DNA content, as a measure of cell number, suggested that cellularity decreased over time in all hydrogel formulations (Figure 3b). DNA content significantly decreased from day 1 to day 7 in CS-MA and Ch-MA hydrogels and by day 14 in PEG controls. DNA content remained largely consistent across hydrogel composition with no significant differences across the three hydrogel compositions on days 1, 7 and 14; however, on day 21, PEG control hydrogels experienced a significant decrease in DNA content, compared to MSCs in both CS-MA and Ch-MA compositions.

3.5. Gene Expression of Encapsulated MSCs

In the presence of chondrogenic medium, human MSCs encapsulated in nonsulfated 50% Ch-MA hydrogels significantly upregulated gene expression of the cartilaginous ECM molecules collagen II and aggrecan on days 7, 14, and 21, over 50% CS-MA hydrogels (Figure 4a-b). MSCs in 50% Ch-MA expressed 115 ± 100 fold upregulation of collagen II expression on day 21, compared to only 0.014 ± 0.009 in 50% CS-MA and 9.9 ± 19.2 fold in PEG controls (Figure 4a). Similarly, aggrecan expression in Ch-MA gels was upregulated 23.1 ± 7.9 fold on day 21 in the presence of TGF- β 1, compared to 9.0 ± 3.1 in CS-MA and 6.5 ± 1.2 in PEG (Figure 4b). Cartilaginous transcription factor SOX9 experienced slight upregulation on day 7 only in 50% Ch-MA with 1.50 ± 0.28 fold regulation, while CS-MA

only expressed 0.48 ± 0.39 fold regulation (Figure 4c). Collagen X, an ECM marker of hypertrophic chondrocytes, was also significantly upregulated in Ch-MA hydrogels over CS-MA controls in chondrogenic medium on days 7, 14, and 21 (Figure 4d). MSCs in 50% Ch-MA exhibited large upregulation of collagen X of $3,200 \pm 1,020$ fold on day 21, compared to 483 ± 274 in CS-MA. In the absence of chondrogenic medium, however, MSCs encapsulated in 50% Ch-MA hydrogels did not upregulate any of the chondrogenic markers analyzed here (data not shown).

Markers for other tissues including fibroblastic marker collagen I, osteoblastic marker osteocalcin, myofibroblastic marker MyoD, and adipocytic marker PPAR- γ 2 were also examined. Unexpectedly, collagen I was upregulated over the course of this study (Figure 4e). MSCs in CS-MA and Ch-MA hydrogels exhibited 53.3 ± 6.5 and 56.2 ± 4.3 fold upregulation of collagen I by day 21, respectively, and PEG controls gels expressed 7.6 ± 1.1 fold regulation. Osteocalcin and MyoD, however, did not exhibit significant differences across any of the hydrogel types, while PPAR- γ 2 did not amplify within 40 PCR cycles, indicating low PPAR- γ 2 expression at all time points (data not shown).

3.6. ECM Production by Encapsulated MSCs

Immunostaining for ECM production demonstrated that greater ECM production overall was observed in GAG-containing hydrogels over PEG control hydrogels, which showed relatively little staining for ECM (Figure 5). While some accumulation of cartilaginous ECM collagen II and aggrecan and hypertrophic chondrocyte marker collagen X was observed pericellularly over 21 days, staining was generally weak and clear differences were not apparent between CS-MA and Ch-MA formulations (Figure 5b-d). Deposition of collagen I appeared to persist over time, but few differences were observed between CS-MA and Ch-MA hydrogels (Figure 5e). IgG1 and IgM isotype controls demonstrated little non-specific background staining (Figure 5a).

3.7. TGF- β 1 Pull-Down by CS-MA/Ch-MA-Containing Hydrogels

In pull-down (depletion) studies from solution, all hydrogels exhibited significant pull-down of TGF- β 1, compared to blank wells without gels, where less TGF- β 1 remaining in solution indicated greater pull-down; however, pull-down in 50% CS-MA hydrogels was significantly greater than less sulfated hydrogel formulations (Figure 6). 50% CS-MA depleted $55.9 \pm 1.5\%$ of available TGF- β 1 out of solution (equivalent to 44.1% remaining in solution), compared to blanks. Ch-MA and PEG control hydrogels, on the other hand, experienced the least pull-down of TGF- β 1 with $29.4 \pm 2.7\%$ and $33.8 \pm 3.0\%$ depletion, respectively.

In the presence of 0.5 M NaCl, pull-down of TGF- β 1 by 50% Ch-MA hydrogels significantly decreased, while pull-down by 50% CS-MA did not decrease. Depletion by 50% Ch-MA hydrogels decreased to $14.8 \pm 2.0\%$ in 0.5 M NaCl. Additionally, in the presence of 10 mg/mL soluble CS, a similar response was observed, where pull-down by 50% Ch-MA hydrogel was significantly decreased to $16.4 \pm 1.8\%$ depletion; however, pull-down by crosslinked 50% CS-MA hydrogels did not decrease. TGF- β 1 pull-down by PEG control hydrogels, on the other hand, remained unchanged in 0.5 M NaCl, compared to in 1% BSA, with $33.1 \pm 3.5\%$ depletion, while soluble CS resulted in a slight decrease in depletion by PEG controls to $26.7 \pm 5.5\%$.

4. Discussion

Together, these studies demonstrated that CS could be successfully desulfated, CS and chondroitin materials could be crosslinked to form hydrogels, CS-based hydrogels

sequestered positively charged proteins including TGF- β 1 in a sulfation-dependent manner, and that MSCs upregulated expression of chondrogenic gene markers when encapsulated in nonsulfated chondroitin materials in the presence of TGF- β 1. Chondroitin sulfate was successfully desulfated by acidic methanol treatment after 7 days to yield chondroitin. DMMB assay, FTIR spectroscopy, and SAX-HPLC collectively indicated that chondroitin was ~98.5% nonsulfated after treatment (Figure 1b-c, Table III). SAX-HPLC estimated that desulfation of CS resulted in a 2.3-fold reduction in negative charge density, due to removal of negatively charged sulfates; however, due to the presence of carboxylates in the GAG backbone, chondroitin chains remained moderately negatively charged with approximately one negatively charged group per disaccharide unit.

After 3 days of treatment, CS only underwent ~80% desulfation; however, extended treatment with acidic methanol after 7 days resulted in nearly complete desulfation of CS, implying that desulfation of CS was time-dependent with increasing desulfation over time (Figure 1b). These results suggested that a wide range of degrees of sulfation could be produced by increasing or decreasing the treatment time up to 7 days. In conjunction with various chemical methods of oversulfation [14, 29], a diverse assortment of sulfated materials with varying degrees of charge could be developed from a single GAG structure. These results present desulfated chondroitin materials as a highly controlled system to investigate of the role of sulfation and resulting negative charge density, without altering the chemical composition of the remaining polysaccharide backbone, and these findings present a versatile technology that can be adapted for other GAGs to further examine electrostatic interactions with a variety of signaling molecules.

SEC-HPLC analysis, however, suggested that desulfated chondroitin chains may be of shorter average length than the starting CS chains, and that acidic methanol treatment for 7 days may result in some depolymerization for chondroitin materials, possibly due to hydrolysis at low pH. The reaction parameters of the acidic methanol treatment may require optimization by altering the reaction time or the concentration of reagents to limit potential degradation of chondroitin. Additionally, other chemical procedures have been developed to desulfate CS, and these techniques could be explored as alternative chemistries to prevent degradation of chondroitin chains [30, 31]. Nevertheless, while molecular weight is likely important in material properties and growth factor interactions, methacrylation and crosslinking of CS-MA and Ch-MA materials, as performed in these studies, were expected to mitigate potential differences in molecular mass, as GAG chains were crosslinked together and became immobilized with a highly crosslinked polymer network. Methacrylation of CS and chondroitin with GMA permitted crosslinking of GAG materials by free radical initiation, and ^1H NMR analysis suggested that CS-MA and Ch-MA were similarly methacrylated, approximately once per every 4 disaccharides on average, indicating that similar crosslinking density of the two GAGs may result in comparable final hydrogels but with differing degrees of sulfation and charge.

Crosslinking of CS-MA and Ch-MA chains to form bulk hydrogels also did not prevent degradation of the hydrogel networks by chondroitinase (data not shown). Retention of enzymatic degradation of these biomaterials is especially important, as cell-secreted chondroitinase enzyme could facilitate degradation of CS-MA and Ch-MA materials and permit enzymatically responsive growth factor release or localized remodeling of the surrounding GAG matrix. Conversely, with no chondroitinase present, staining with DMMB after 5 days of swelling in PBS indicated that CS was retained within the crosslinked hydrogel scaffolds with increasing CS content clearly visible (Figure 2b), demonstrating that the GAGs remained covalently linked within the hydrogel network over relatively long periods of time in the absence of any degradative agent.

Desulfation of CS was found to alter the ability of CS to both retain and release a positively charged model protein histone *in vitro*. In histone release studies, 100% CS-MA materials were found to exhibit greater retention of histone, while decreasing the CS content (with a reduction in sulfation and negative charge) resulted in significantly greater release (Figure 2d), suggesting that sulfation of CS plays a fundamental role in the retention of positively charged proteins. Additionally, protein release even after the initial burst release varied with the relative degree of sulfation with more rapid histone release observed from the less sulfated 10% CS-MA and 100% Ch-MA hydrogels, compared to the more highly sulfated 100% CS-MA and 50% CS-MA compositions, and these results indicated that desulfation of CS-based materials may have considerable application to adjust the long term protein release kinetics in a sulfation-dependent manner. These differences in histone retention and release were observed in spite of similar degrees of swelling between 100% CS-MA and 100% Ch-MA hydrogels (Figure 2c), suggesting that the charged histone protein was retained via electrostatic interactions with the negatively charged sulfates in CS, rather than simply physical entrapment within the hydrogel network.

In particular, as naturally-derived cartilaginous ECM, sulfated and desulfated CS-based materials may be promising tools to sequester or deliver TGF- β 1 for cartilage regeneration and repair, so human MSCs were encapsulated in CS- and chondroitin-containing PEG hydrogels as a model system, similar to previously reported CS-based systems of chondrogenic differentiation [15, 16, 18], to examine the roles of sulfation and growth factor interactions in localized cell response. MSC encapsulation studies demonstrated that viable MSCs remained dispersed throughout PEG-based hydrogels containing 50% GAG after 3 weeks of culture *in vitro* (Figure 3), and that nonsulfated chondroitin materials upregulated gene expression of chondrogenic markers by human MSCs, compared to CS materials, when cultured in chondrogenic medium (Figure 4). MSCs encapsulated in PEG-based hydrogels containing 50% Ch-MA exhibited significantly greater gene expression of collagen II and aggrecan than MSCs in 50% CS-MA gels after 21 days of culture in chondrogenic medium containing TGF- β 1 and greater early expression of SOX9 on day 7 only, suggesting that nonsulfated chondroitin materials may promote greater chondrogenic differentiation over CS.

Because PEG hydrogels containing 50% Ch-MA swelled significantly less than 50% CS-MA (Table I), in these experiments, PEG hydrogels that swelled similarly to 50% Ch-MA hydrogels were used as swelling controls. MSCs have been shown to regulate differentiation in response to both substrate stiffness and pore size *in vitro* [32, 33]; therefore, PEG swelling controls were used as approximate controls for material stiffness and porosity/mesh size in the absence of charged polysaccharides [34, 35]. Atomic force microscopy (AFM) measurements of hydrogel stiffness indicated that the elastic moduli of all three hydrogel compositions used for MSC encapsulation were not significantly different from each other (data not shown), suggesting that substrate stiffness did not play a role in modulating the differentiation response. In these experiments, MSCs in uncharged PEG control hydrogels expressed significant upregulation of collagen II, aggrecan, and SOX9 gene expression over 50% CS-MA hydrogels after 3 weeks; however, gene expression of chondrogenic markers remained less than that observed in Ch-MA materials, suggesting that upregulation in 50% Ch-MA hydrogels was not solely due to differences in swelling properties. Additionally, in the absence of TGF- β 1 and dexamethasone, chondroitin materials did not exhibit significant upregulation of gene expression for cartilaginous markers, indicating that nonsulfated chondroitin materials alone were not sufficient to upregulate gene expression of cartilaginous ECM by MSCs and that upregulation of chondrogenic markers may be dependent on interactions between the chondroitin material and chondrogenic cues from exogenously supplemented TGF- β 1.

While MSCs significantly upregulated expression of chondrogenic markers in 50% Ch-MA hydrogels, gene expression of collagen I, which is normally produced in high amounts by undifferentiated MSCs but is not normally present in hyaline cartilage [36], did not decrease and actually appeared to increase over the time course of these experiments (Figure 4e). Additionally, gene expression of collagen X, an ECM marker of hypertrophic chondrocytes, was significantly upregulated in 50% Ch-MA hydrogels, compared to CS-MA and PEG control gels (Figure 4d). As key differentiation markers of osteogenic, myofibroblastic, and adipocytic phenotypes were not significantly regulated in these studies (data not shown), these gene expression results appear to be more representative of fibrochondrocytic or hypertrophic chondrocyte phenotypes. Upregulation of collagen X expression is consistent with previous studies of *in vitro* chondrogenesis [37, 38], and hypertrophy of chondrogenic MSCs remains a key challenge to differentiation *in vitro*. While further optimization of culture conditions are needed to assure a non-hypertrophic phenotype before these constructs could be translated *in vivo*, such experiments are beyond the scope of the current study, which focused on the effects of the different forms of chondroitin on chondrogenesis.

Despite significant differences in gene expression between MSCs encapsulated in Ch-MA and CS-MA hydrogels, differences in ECM deposition were not as apparent in immunostaining of the cell-laden hydrogel sections. In general, greater ECM deposition was observed pericellularly in GAG-based CS-MA and Ch-MA materials than PEG control gels, which exhibited limited staining for ECM overall (Figure 5); however, notable differences in collagen II and aggrecan deposition were not evident between Ch-MA and CS-MA hydrogels. These results suggested that MSCs may have lacked sufficient extracellular space for significant matrix production in these tightly crosslinked hydrogel scaffolds. Chondrogenic human MSCs may not produce sufficient amounts of chondroitinase enzyme to effectively degrade the GAG network, thus inhibiting the deposition of cartilaginous ECM by encapsulated MSCs. Our results are consistent with other findings using human MSCs in CS/PEG hydrogels that demonstrated only pericellular matrix deposition and little material degradation after 14 days [18]. Therefore, in the future, incorporation of hydrolytically- or enzymatically-degradable moieties [39, 40] may be necessary support greater ECM deposition by human MSCs in Ch-MA and CS-MA materials.

To examine the ability of TGF- β 1 to bind and interact with GAG-based hydrogels, “pull-down” of soluble TGF- β 1 out of solution was explored in CS-MA and Ch-MA hydrogels. This system is comparable to culture of GAG-based constructs in chondrogenic media, in which hydrogels may be able to sequester TGF- β 1 to promote signaling and differentiation. Pull-down experiments in these gels demonstrated that 50% CS-MA hydrogels sequestered soluble TGF- β 1, “pulling” it out of solution in 1% BSA and retaining it within the hydrogel network (Figure 6). Decreasing the sulfation of the GAG matrix reduced the observed interaction with TGF- β 1 in 50% Ch-MA hydrogels, indicating that sulfation and charge play important roles in facilitating depletion of TGF- β 1. Interestingly, increasing the ionic strength of the buffer with an additional 0.5 M NaCl or competitively inhibiting sequestration with 10 mg/mL soluble CS significantly inhibited pull-down of TGF- β 1 by 50% Ch-MA, while pull-down by 50% CS-MA did not appear to decrease. Inhibition of pull-down in the 50% Ch-MA hydrogels in 0.5 M NaCl suggested that the moderate negative charge of chondroitin may retain a weaker electrostatic interaction with TGF- β 1 than the corresponding CS hydrogels. Additionally, the absence of competitive inhibition by 10 mg/mL soluble CS in 50% CS-MA suggested that TGF- β 1 may preferentially bind crosslinked CS-MA hydrogels over soluble CS chains in solution, due to the immobilization of negatively charged CS chains in close proximity by crosslinking of these hydrogel materials. In fact, this multivalent interaction may be more comparable to CS proteoglycans, which are the native form that GAGs typically sequester proteins *in vivo*, with GAGs covalently linked to a protein core and anchored in close proximity [41].

These pull-down experiments suggest that CS-based materials may sequester TGF- β 1 from chondrogenic medium, thus modulating signaling within GAG-based scaffolds. Although enhanced upregulation of chondrogenic markers in Ch-MA materials over CS-MA hydrogels may appear to contradict this hypothesis, the high negative charge density of CS in these materials could potentially decrease TGF- β 1 activity or inhibit transport within the hydrogel network; therefore, removal of sulfate groups may promote MSC differentiation via enhanced growth factor signaling. The highly charged CS matrix may also prevent transport of other cell-secreted signals within the hydrogel, effectively inhibiting intercellular communication, which plays important roles in supporting chondrogenic differentiation and maintaining a chondrocytic phenotype [10]. In addition, while these results suggest that decreasing the degree of sulfation in GAG-based hydrogels may enhance chondrogenic differentiation of MSCs over highly sulfated CS through interaction with TGF- β 1, it is important to consider that sulfation and charge may also alter the extracellular microenvironment through related differences in osmotic swelling pressure [42, 43] or through various interactions with the ECM, cell surface receptors, or other signaling molecules to influence MSC gene expression in response to soluble chondrogenic cues, independent of biomaterial sequestration of TGF- β 1. For example, hyaluronan, the unsulfated GAG which has a similar chemical structure to desulfated chondroitin, possesses unique signaling activity, including known cell surface receptors like CD44 and receptor for hyaluronan-mediated motility (RHAMM) [44, 45], suggesting that desulfated chondroitin materials may potentially be capable of similar receptor interactions that are currently unidentified. Therefore, to fully elucidate the role of GAG-based materials in promoting chondrogenic differentiation, further investigation would be required to determine the cellular interactions with CS/chondroitin and downstream signals that result in an enhanced chondrogenic response.

5. Conclusions

These studies have demonstrated that crosslinkable desulfated chondroitin is a promising biomaterial tool to investigate the role of sulfation in GAG interactions with growth factors for protein sequestration and delivery, as well as stem cell differentiation. Hydrogels containing nonsulfated Ch-MA exhibited more rapid release of positively charged protein (in a dose-dependent manner) compared to CS-MA, suggesting that these materials may provide a means to control growth factor release based on the degree of sulfation. CS-MA hydrogels were also able to sequester greater amounts of the positively charged chondrogenic growth factor TGF- β 1 than Ch-MA materials, further supporting the idea that controlling sulfation of the material plays an important role in facilitating the interactions between charged growth factors and GAG-based biomaterials. When CS and chondroitin were incorporated into PEG-based hydrogels, encapsulated human MSCs demonstrated enhanced gene expression of chondrogenic markers in chondroitin-containing materials in the presence of TGF- β 1, compared to CS and PEG-only hydrogels, suggesting that sulfation may also play an important role in regulating MSC response to soluble differentiation cues. Such results present this newly-developed family of crosslinkable CS and nonsulfated chondroitin materials as a valuable platform to regulate GAG sulfation in a precise manner in order to control biomaterial interactions with charged growth factors. Thus, these findings have significant implications in the development of affinity-based biomaterials to regulate and control growth factor delivery, release, and signaling for tissue repair in a wide variety of clinical applications.

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References

- [1]. Lamberg SI, Stoolmiller AC. Glycosaminoglycans. A biochemical and clinical review. *J Invest Dermatol.* 1974; 63:433–49. [PubMed: 4139221]
- [2]. Lindahl U, Hook M. Glycosaminoglycans and their binding to biological macromolecules. *Annu Rev Biochem.* 1978; 47:385–417. [PubMed: 354500]
- [3]. Zafiropoulos A, Fthenou E, Chatzinikolaou G, Tzanakakis GN. Glycosaminoglycans and PDGF signaling in mesenchymal cells. *Connect Tissue Res.* 2008; 49:153–6. [PubMed: 18661332]
- [4]. Rider CC. Heparin/heparan sulphate binding in the TGF-beta cytokine superfamily. *Biochem Soc Trans.* 2006; 34:458–60. [PubMed: 16709187]
- [5]. Lin XH. Functions of heparan sulfate proteoglycans in cell signaling during development. *Development.* 2004; 131:6009–21. [PubMed: 15563523]
- [6]. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature.* 2007; 446:1030–7. [PubMed: 17460664]
- [7]. Pike DB, Cai S, Pomraning KR, Firpo MA, Fisher RJ, Shu XZ, et al. Heparin-regulated release of growth factors in vitro and angiogenic response in vivo to implanted hyaluronan hydrogels containing VEGF and bFGF. *Biomaterials.* 2006; 27:5242–51. [PubMed: 16806456]
- [8]. Sakiyama-Elbert SE, Hubbell JA. Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix. *J Control Release.* 2000; 69:149–58. [PubMed: 11018553]
- [9]. Edelman ER, Mathiowitz E, Langer R, Klagsbrun M. Controlled and modulated release of basic fibroblast growth factor. *Biomaterials.* 1991; 12:619–26. [PubMed: 1742404]
- [10]. DeLise AM, Fischer L, Tuan RS. Cellular interactions and signaling in cartilage development. *Osteoarthritis Cartilage.* 2000; 8:309–34. [PubMed: 10966838]
- [11]. Shibata S, Fukada K, Imai H, Abe T, Yamashita Y. In situ hybridization and immunohistochemistry of versican, aggrecan and link protein, and histochemistry of hyaluronan in the developing mouse limb bud cartilage. *J Anat.* 2003; 203:425–32. [PubMed: 14620382]
- [12]. French MM, Smith SE, Akanbi K, Sanford T, Hecht J, Farach-Carson MC, et al. Expression of the heparan sulfate proteoglycan, perlecan, during mouse embryogenesis and perlecan chondrogenic activity in vitro. *J Cell Biol.* 1999; 145:1103–15. [PubMed: 10352025]
- [13]. Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K. Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. *J Biol Chem.* 2002; 277:43707–16. [PubMed: 12221095]
- [14]. Hintze V, Miron A, Moeller S, Schnabelrauch M, Wiesmann HP, Worch H, et al. Sulfated hyaluronan and chondroitin sulfate derivatives interact differently with human transforming growth factor-beta1 (TGF-beta1). *Acta Biomater.* 2012; 8:2144–52. [PubMed: 22426137]
- [15]. Varghese S, Hwang NS, Canver AC, Theprungsirikul P, Lin DW, Elisseeff J. Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells. *Matrix Biol.* 2008; 27:12–21. [PubMed: 17689060]
- [16]. Nguyen LH, Kudva AK, Guckert NL, Linse KD, Roy K. Unique biomaterial compositions direct bone marrow stem cells into specific chondrocytic phenotypes corresponding to the various zones of articular cartilage. *Biomaterials.* 2011; 32:1327–38. [PubMed: 21067807]
- [17]. Chen WC, Yao CL, Chu IM, Wei YH. Compare the effects of chondrogenesis by culture of human mesenchymal stem cells with various type of the chondroitin sulfate C. *J Biosci Bioeng.* 2011; 111:226–31. [PubMed: 21035392]

- [18]. Steinmetz NJ, Bryant SJ. Chondroitin sulfate and dynamic loading alter chondrogenesis of human MSCs in PEG hydrogels. *Biotechnol Bioeng.* 2012
- [19]. Schubert, M. Chondroitin from chondroitin sulfate. In: Whistler, RL.; BeMiller, JN.; Wolfrom, ML., editors. *Methods in Carbohydrate Chemistry, Volume V: General Polysaccharides.* Academic Press; New York: 1965. p. 109-10.
- [20]. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta.* 1986; 883:173–7. [PubMed: 3091074]
- [21]. Baier, Leach J.; Bivens, KA.; Patrick, CW., Jr.; Schmidt, CE. Photocrosslinked hyaluronic acid hydrogels: natural, biodegradable tissue engineering scaffolds. *Biotechnol Bioeng.* 2003; 82:578–89. [PubMed: 12652481]
- [22]. DeLong SA, Moon JJ, West JL. Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration. *Biomaterials.* 2005; 26:3227–34. [PubMed: 15603817]
- [23]. Jo S, Shin H, Shung AK, Fisher JP, Mikos AG. Synthesis and characterization of oligo(poly(ethylene glycol) fumarate) macromer. *Macromolecules.* 2001; 34:2839–44.
- [24]. Brink KS, Yang PJ, Temenoff JS. Degradative properties and cytocompatibility of a mixed-mode hydrogel containing oligo[poly(ethylene glycol)fumarate] and poly(ethylene glycol)dithiol. *Acta Biomater.* 2009; 5:570–9. [PubMed: 18948068]
- [25]. Yamagata T, Saito H, Habuchi O, Suzuki S. Purification and properties of bacterial chondroitinases and chondrosulfatases. *J Biol Chem.* 1968; 243:1523–35. [PubMed: 5647268]
- [26]. Ahn SJ, Costa J, Emanuel JR. PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. *Nucleic Acids Res.* 1996; 24:2623–5. [PubMed: 8692708]
- [27]. Ruijter JM, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, van den Hoff MJ, et al. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 2009; 37:e45. [PubMed: 19237396]
- [28]. Box GEP, Cox DR. An analysis of transformations. *J R Stat Soc Series B Stat Methodol.* 1964; 26:211–52.
- [29]. Nagasawa K, Uchiyama H, Wajima N. Chemical sulfation of preparations of chondroitin 4-sulfate and 6-sulfate, and dermatan sulfate. Preparation of chondroitin sulfate E-like materials from chondroitin 4-sulfate. *Carbohydr Res.* 1986; 158:183–90.
- [30]. Nagasawa K, Inoue Y, Tokuyasu T. Improved method for the preparation of chondroitin by solvolytic desulfation of chondroitin sulfates. *J Biochem.* 1979; 86:1323–9. [PubMed: 521436]
- [31]. Navarro DA, Flores ML, Stortz CA. Microwave-assisted desulfation of sulfated polysaccharides. *Carbohydrate Polymers.* 2007; 69:742–7.
- [32]. Park JS, Chu JS, Tsou AD, Diop R, Tang Z, Wang A, et al. The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta. *Biomaterials.* 2011; 32:3921–30. [PubMed: 21397942]
- [33]. Kasten P, Beyen I, Niemeyer P, Luginbuhl R, Böhner M, Richter W. Porosity and pore size of beta-tricalcium phosphate scaffold can influence protein production and osteogenic differentiation of human mesenchymal stem cells: an in vitro and in vivo study. *Acta Biomater.* 2008; 4:1904–15. [PubMed: 18571999]
- [34]. Canal T, Peppas NA. Correlation between Mesh Size and Equilibrium Degree of Swelling of Polymeric Networks. *J Biomed Mater Res A.* 1989; 23:1183–93.
- [35]. Temenoff JS, Athanasiou KA, LeBaron RG, Mikos AG. Effect of poly(ethylene glycol) molecular weight on tensile and swelling properties of oligo(poly(ethylene glycol) fumarate) hydrogels for cartilage tissue engineering. *J Biomed Mater Res.* 2002; 59:429–37. [PubMed: 11774300]
- [36]. Steck E, Bertram H, Abel R, Chen BH, Winter A, Richter W. Induction of intervertebral disc-like cells from adult mesenchymal stem cells. *Stem Cells.* 2005; 23:403–11. [PubMed: 15749935]
- [37]. Ichinose S, Yamagata K, Sekiya I, Muneta T, Tagami M. Detailed examination of cartilage formation and endochondral ossification using human mesenchymal stem cells. *Clin Exp Pharmacol Physiol.* 2005; 32:561–70. [PubMed: 16026516]
- [38]. Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, et al. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with

- calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum.* 2006; 54:3254–66. [PubMed: 17009260]
- [39]. Qiu Y, Lim JJ, Scott L Jr, Adams RC, Bui HT, Temenoff JS. PEG-based hydrogels with tunable degradation characteristics to control delivery of marrow stromal cells for tendon overuse injuries. *Acta Biomater.* 2011; 7:959–66. [PubMed: 21056127]
- [40]. Gobin AS, West JL. Cell migration through defined, synthetic ECM analogs. *FASEB J.* 2002; 16:751–3. [PubMed: 11923220]
- [41]. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. *FASEB J.* 1992; 6:861–70. [PubMed: 1740236]
- [42]. Villanueva I, Gladem SK, Kessler J, Bryant SJ. Dynamic loading stimulates chondrocyte biosynthesis when encapsulated in charged hydrogels prepared from poly(ethylene glycol) and chondroitin sulfate. *Matrix Biol.* 2010; 29:51–62. [PubMed: 19720146]
- [43]. Villanueva I, Bishop NL, Bryant SJ. Medium osmolarity and pericellular matrix development improves chondrocyte survival when photoencapsulated in poly(ethylene glycol) hydrogels at low densities. *Tissue Eng Part A.* 2009; 15:3037–48. [PubMed: 19331581]
- [44]. Knudson CB, Knudson W. Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *FASEB J.* 1993; 7:1233–41. [PubMed: 7691670]
- [45]. Bourguignon LY, Singleton PA, Zhu H, Zhou B. Hyaluronan promotes signaling interaction between CD44 and the transforming growth factor beta receptor I in metastatic breast tumor cells. *J Biol Chem.* 2002; 277:39703–12. [PubMed: 12145287]

Figure 1.

Desulfation of chondroitin sulfate materials to form chondroitin. (a) Chondroitin sulfate was desulfated in acidic methanol for up to 7 days to form a methyl ester of chondroitin. The methyl ester was demethylated in potassium hydroxide for 24 hours to yield chondroitin. (b) Dimethylmethylene blue (DMMB) assay standards for CS showed a linear trend with positive slope, indicating the presence of sulfates; however, the standard for the chondroitin product from CS desulfated for 7 days showed zero slope, indicating the absence of sulfate groups in chondroitin. CS experienced incomplete desulfation after 3 days. (c) Fourier transform infrared (FTIR) spectroscopy showed the absence of sulfate peaks from 1100-1250 cm^{-1} (in black box) in chondroitin materials, compared to CS, without modification of the remaining chemical bonds in CS.

Figure 2.

CS-MA/Ch-MA hydrogels with varying degrees of sulfation affect retention of a positively charged model protein. (a) Chondroitin sulfate was methacrylated with glycidyl methacrylate in 50:50 acetone:ddH₂O for 24 hours in the presence of triethylamine to form chondroitin sulfate methacrylate. (b) DMMB staining indicated increasing degrees of sulfation in hydrogels from 0% to 100% CS-MA content (remainder of formulation was nonsulfated Ch-MA) 5 days after fabrication. Scale bars = 2 mm. (c) Mixtures of 10% CS-MA/90% Ch-MA and 50% CS-MA/50% Ch-MA hydrogels swelled significantly less than 100% Ch-MA and 100% CS-MA, while swelling of 100% Ch-MA and CS-MA materials was not significantly different. * indicates significantly lower fold swelling than 100% Ch-MA and 100% CS-MA ($p < 0.05$). (d) 100% CS-MA hydrogels demonstrated greatest retention of positively charged histone over 8 days, while 50% CS-MA/50% Ch-MA gels exhibited greater release, and 100% Ch-MA and 10% CS-MA/90% Ch-MA materials displayed the greatest release. # indicates less release than 100% Ch-MA. + indicates significantly less release than 10% CS-MA/90% Ch-MA ($p < 0.05$). § indicates significantly less release than 50% CS-MA/50% Ch-MA ($p < 0.05$).

Figure 3.

Viability and cellularity of human MSCs encapsulated in 50% CS-MA and 50% Ch-MA hydrogels (remainder of hydrogel formulation was PEG-DA/OPF) in chondrogenic medium.

(a) Human MSCs appeared largely viable in all hydrogel formulations over 21 days of culture. Scale bars = 100 μ m. (b) DNA content, as a measure of cellularity, decreased over time with a significant decrease observed between day 1 and day 7 in CS-MA and Ch-MA hydrogels and by day 14 in PEG controls. Additionally, a significant decrease in DNA content was measured in PEG control hydrogels on day 21 compared to other hydrogel types. * indicates significantly less DNA than day 1 ($p < 0.05$). # indicates significantly less DNA than 50% Ch-MA at the same time point ($p < 0.05$). + indicates significantly less DNA than 50% CS-MA at the same time point ($p < 0.05$).

Figure 4.

Gene expression of chondrocytic markers by MSCs in 50% CS-MA and 50% Ch-MA hydrogels in chondrogenic medium. MSCs in 50% Ch-MA hydrogels significantly upregulated gene expression of (a) collagen II and (b) aggrecan after 7, 14, and 21 days of culture in chondrogenic medium, and (c) SOX9 on day 7 only, compared to 50% CS-MA. (Collagen II expression on day 21 is depicted on separate axes using a different vertical scale.) (d) Hypertrophic chondrocyte marker collagen X was also significantly upregulated in 50% Ch-MA gels on days 7, 14, and 21 over 50% CS-MA. (e) MSCs upregulated collagen I gene regulation in 50% CS-MA and 50% Ch-MA materials as well, compared to PEG controls, on days 7, 14, and 21. * indicates significantly greater than 50% CS-MA at same time point ($p < 0.05$). # indicates significantly greater than PEG controls at same time point ($p < 0.05$). + indicates significantly greater than same hydrogel type at day 1 ($p < 0.05$).

Figure 5.

Immunostaining for ECM deposition by MSCs in 50% CS-MA and 50% Ch-MA hydrogels in chondrogenic medium. (a) Representative negative controls stained with isotype control antibodies showed little background staining on day 21. (b) Collagen II, (c) aggrecan, (d) collagen X, and (e) collagen I were produced in small quantities by MSCs in CS-MA and Ch-MA hydrogels, compared to relatively little deposition in PEG gels. Little difference was observed between 50% CS-MA and 50% Ch-MA. Cell nuclei = blue. Scale bars = 100 m.

Figure 6.

TGF- β 1 pull-down by CS-MA and Ch-MA hydrogels with varying degrees of sulfation. All hydrogels demonstrated significant pull-down compared to blanks ($p < 0.05$), represented as 1, where less TGF- β 1 remaining in solution indicated greater pull-down. 50% CS-MA hydrogel exhibited the greatest pull-down of soluble TGF- β 1 out of solution, while nonsulfated 50% Ch-MA and PEG control hydrogels exhibited the least depletion in 1% BSA. Incubation in 0.5 M NaCl and 10 mg/ml soluble CS significantly decreased depletion by 50% Ch-MA, but not 50% CS-MA hydrogels. * indicates significantly greater pull-down than 50% Ch-MA ($p < 0.05$). # indicates significantly greater pull-down than PEG controls ($p < 0.05$). + indicates significantly less pull-down than the same hydrogel type in 1% BSA ($p < 0.05$).

Table I

Hydrogel formulations with 50 wt% GAG, 25% PEG-DA, 25% OPF 10K

	Mass Ratios of Total Dry Polymer				Fold Swelling
	CS-MA	Ch-MA	PEG-DA	OPF 10K	
50% CS-MA	50%	0%	25%	25%	25.2 ± 1.4
50% Ch-MA	0%	50%	25%	25%	16.9 ± 2.1
PEG Control	0%	0%	60%	40%	17.1 ± 0.5

* indicates significantly lower fold swelling than 50% CS-MA (p 0.05)

Table II

Human primer sequences for quantitative polymerase chain reaction

Target	Primer Sequences (5'-3')	GenBank
Collagen II ($\alpha 1$)	ACCCAATCCAGCAAACGTT	NM_001844
	ATCTGGACGTTGGCAGTGTTG	
Aggrecan	ACAG CTGGG GACATTAGTG G	NM_001135
	GTGGAATGCAGAGGTGGTTT	
SOX9	GCGGAGGAAGTCGGTGAAGAACGGGCA	NM_000346
	TGTGAGCGGGTGATGGGCGGG	
Collagen X ($\alpha 1$)	GGCCCAGCAGGAGCAAAGGG	NM_000493
	GTGGCCCGGTGGGTCCATTG	
Collagen I ($\alpha 2$)	GAAAACATCCCAGCCAAGAA	NM_000089
	GCCAGTCTCCTCATCCATGT	
Osteocalcin (BGLAP)	GTGCAGAGTCCAGCAAAGGT	NM_199173
	AGCAGAGCGACACCCTAGAC	
MyoD	GTCGAGCCTAGACTGCCTGT	NM_002478
	GTATATCGGGTTGGGGTTCG	
PPAR- $\gamma 2$	TCCATGCTGTTATGGGTGAA	NM_015869
	GGGAGTGGTCTTCCATTACG	
GAPDH	GAGTCAACGGATTGGTCGT	NM_002046
	TTGATTTTGGAGGGATCTCG	

Table III

CS and chondroitin disaccharide composition by SAX-HPLC analysis

	Δ UA-GalNAc	Δ UA-GalNAc(6S)	Δ UA-GalNAc(4S)	Δ UA(2S)-GalNAc(6S)	Δ UA-GalNAc(4S,6S)	Δ UA(2S)-GalNAc(4S)
CS	1.3%	57.8%	26.8%	12.7%	0.7%	0.7%
Chondroitin	98.5%	1.5%	nd	Nd	nd	nd

Δ UA = $\Delta^{4,5}$ -unsaturated uronic acid, GalNAc = N-acetylgalactosamine, nd = not detected