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Hydrolytic (In)stability of Methacrylate Esters in Covalently Cross-Linked Hydrogels Based on Chondroitin Sulfate and Hyaluronic Acid Methacrylate

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Cite This: *ACS Omega* 2021, 6, 26302−26310

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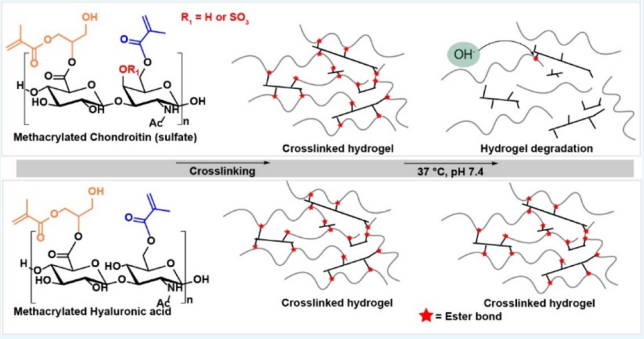
ABSTRACT: Chondroitin sulfate (CS) and hyaluronic acid (HA) methacrylate (MA) hydrogels are under investigation for biomedical applications. Here, the hydrolytic (in)stability of the MA esters in these polysaccharides and hydrogels is investigated. Hydrogels made with glycidyl methacrylate-derivatized CS (CSGMA) or methacrylic anhydride (CSMA) degraded after 2−25 days in a cross-linking density-dependent manner (pH 7.4, 37 °C). HA methacrylate (HAMA) hydrogels were stable over 50 days under the same conditions. CS(G)MA hydrogel degradation rates increased with pH, due to hydroxide-driven ester hydrolysis. Desulfated chondroitin MA hydrogels also degrade, indicating that sulfate groups are not responsible for CS(G)MA's hydrolytic sensitivity (pH 7.0−8.0, 37 °C). This sensitivity is likely because CS(G)MA's *N*-acetyl-galactosamines do not form hydrogen bonds with adjacent glucuronic acid oxygens, whereas HAMA's *N*-acetyl-glucosamines do. This bond absence allows CS(G)MA higher chain flexibility and hydration and could increase ester hydrolysis sensitivity in CS(G)MA networks. This report helps in biodegradable hydrogel development based on endogenous polysaccharides for clinical applications.



1. INTRODUCTION

Chondroitin sulfate (CS) and hyaluronic acid (HA) are structurally related polysaccharides that are present in human extracellular matrix and synovial fluids.[1] Both polysaccharides consist of disaccharide building blocks composed of a glucuronic acid and an amino sugar unit. For CS, this amino sugar is a *N*-acetyl-galactosamine, whereas for HA, it is a *N*-acetyl-glucosamine. In addition to this, CS can be sulfated in several positions.[2−4] Both polysaccharides can be methacrylated through the reaction with, for example, glycidyl methacrylate or methacrylic acid (MA), forming, for instance, chondroitin sulfate glycerol methacrylate (CSGMA), chondroitin sulfate methacrylate (CSMA), or hyaluronic acid methacrylate (HAMA).[2−11] These methacrylated polysaccharides in aqueous medium can be (photo)polymerized to yield hydrogels. Such hydrogels are under investigation for use in drug delivery and tissue engineering due to their biocompatibility and enzymatic biodegradability.[10,12] To mention, both HAMA and CS(G)MA are of interest as a hydrogel-forming polysaccharides in tissue engineering scaffolds, mimicking the environment of cartilage.[1,5,6,13,14] Additionally, CS(G)MA is investigated for its use in neuronal microenvironment engineering.[15] These hydrogel scaffolds have been shown to promote the maintenance of the undifferentiated state of encapsulated neural stem cells by sequestration of endogenous fibroblast growth factor 2.[15] Moreover, cell delivery systems based on CS(G)MA hydrogels have been reported to promote the neuroprotective and regenerative effects of neural stem cells when injected in rats after acute traumatic brain injuries.[15,16]

Considering the significant number of reports on the applications of hydrogels based on CS(G)MA and HAMA, it is important to understand the properties of these hydrogels to fully exploit their pharmaceutical, biotechnological, and biomedical potential. Several parameters are important for such an evaluation. To mention, the hydrogels should, for instance, be sterilizable, biocompatible, and of reasonably uniform and tunable porosity.[17] Another important parameter is hydrogel degradability. Depending on the aimed application, hydrogels can be designed to be stable or to be degraded

Received: June 29, 2021
Accepted: September 20, 2021
Published: September 28, 2021

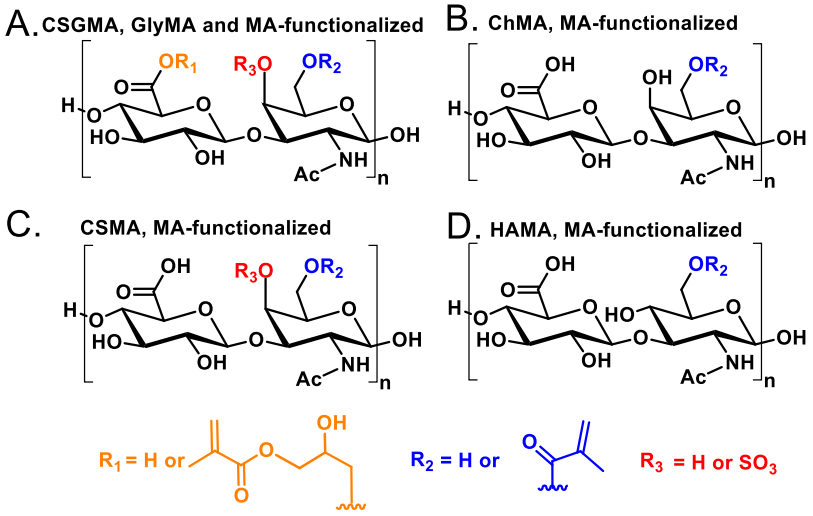


Figure 1. Molecular structures of the derivatized glycosaminoglycans used in this work. (A) CSGMA derivatized with both GlyMA (R_1) and MA (R_2) moieties. (B–D) ChMA, CSMA, and HAMA derivatized with MA moieties.

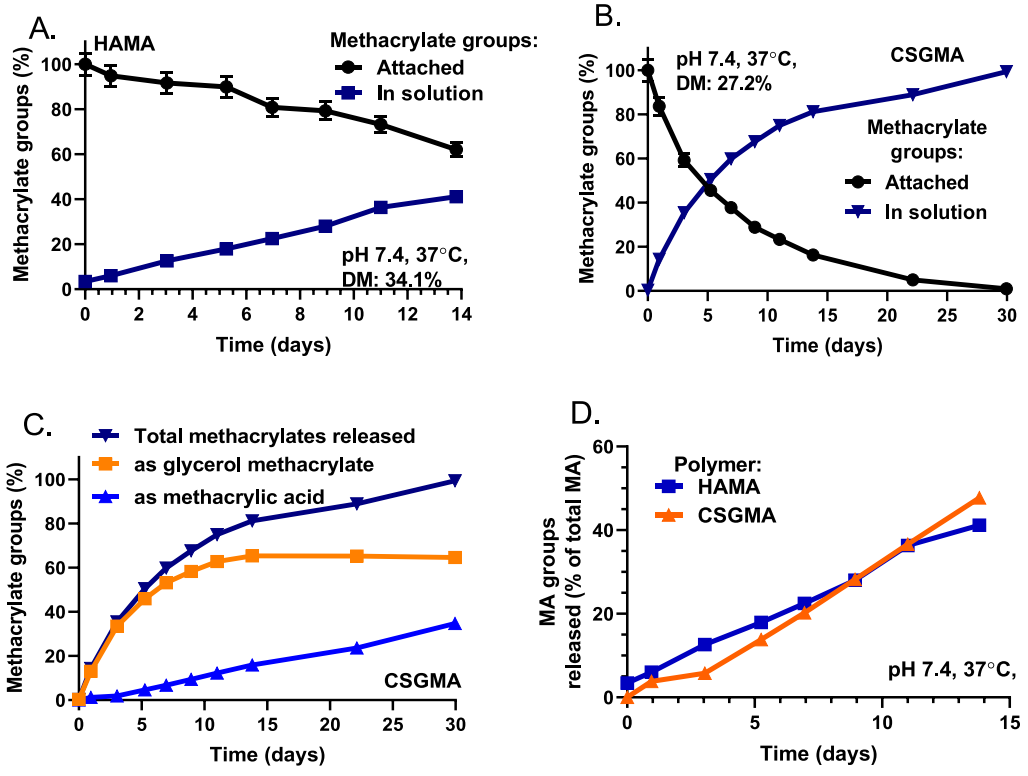


Figure 2. (A) Quantification of attached MA-esters (via ^{1}H NMR, black circles) and total MA-ester hydrolysis, as determined via HPLC (blue squares) from incubated HAMA polymer. (B) Determination of the total combined attached MA and GlyMA-esters to CSGMA (black circles) by ^{1}H NMR and total combined MA and GlyMA-ester hydrolysis over time from the incubated CSGMA polymer, as determined by HPLC quantification. (C) Quantification (HPLC) of the hydrolysis of GlyMA and MA-moieties present on CSGMA under the same conditions as in (B) (blue upward triangles and orange squares, respectively). (D) Determination of MA-ester hydrolysis over time from HAMA and CSGMA by HPLC. Polymer incubation for all experiments depicted was carried out in 50 mM phosphate buffer (154 mM ionic strength, pH 7.4, 37 °C, polymers concentration: 5 mg/mL).

enzymatically or chemically over time.[17] Understanding if and how a clinical candidate hydrogel degrades under certain conditions is of utmost importance as an unfavorable degradation profile might lead to unwanted clinical outcomes. The rate of hydrogel scaffold degradation should ideally be similar in timeframe to the rate of new tissue formation by the cells encapsulated.[18] Typically, degradation timescales are in the range of 2 weeks up to several months, depending on the tissue type and application.[19,20]

Of specific importance in the evaluation of the degradability of HAMA and CS(G)MA hydrogels is the stability of the ester bonds that connect the (cross-linked) MA groups to the polysaccharides. It has previously been found that MA ester linkages and other ester groups present in polysaccharides can be susceptible to hydroxide-mediated hydrolysis under

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ACS Omega 2021, 6, 26302−26310

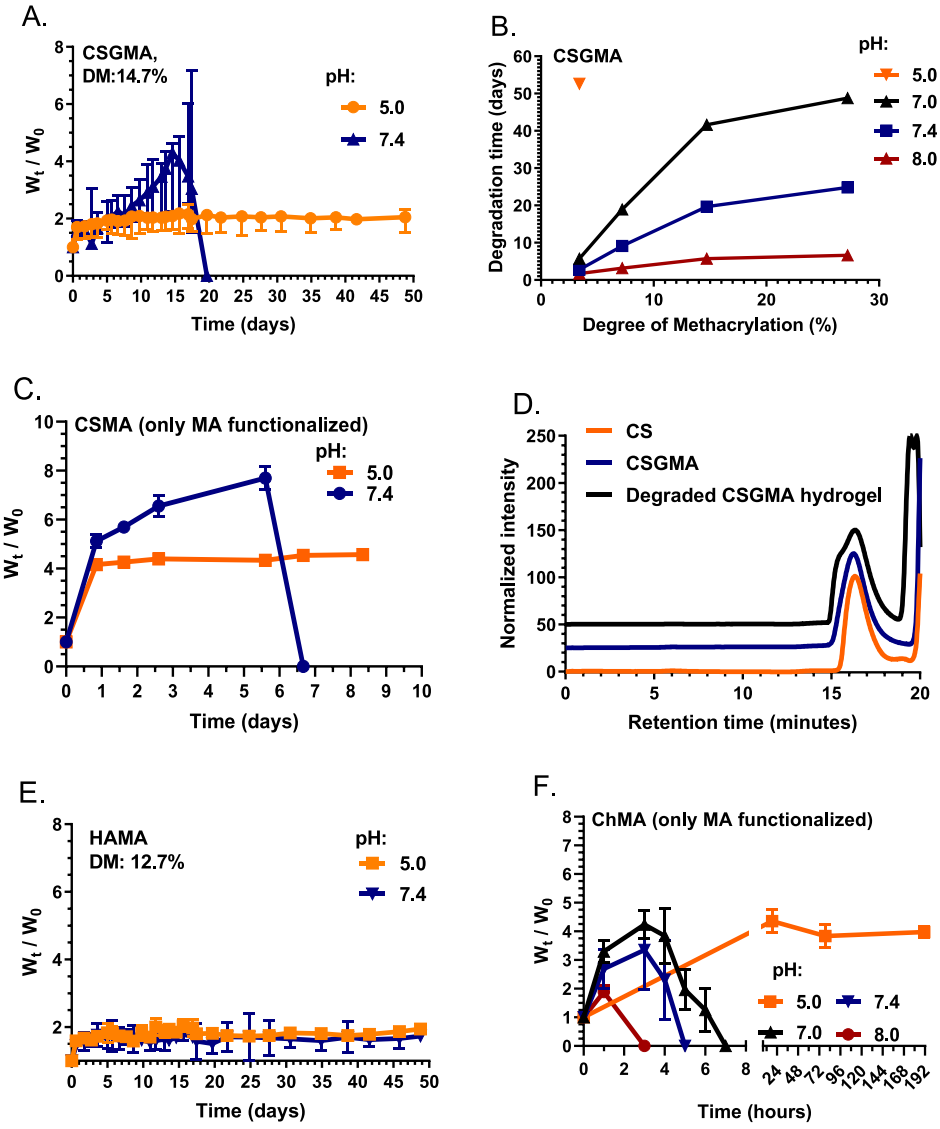

Figure 3. (A) Swelling of 15 wt % CSGMA hydrogels over time. (B) Effects of CSGMA DM and buffer pH on CSGMA hydrogel degradation time. (C) Swelling of MA-functionalized 15 wt % CSMA hydrogels (DM: 6.2%) over time. (D) GPC chromatograms of CS, CSGMA, and liquefied CSGMA hydrogel (DM: 14.7%) incubated in a pH 7.4 buffer at 37 °C for 20 days. (E) Swelling of 15 wt % HAMA hydrogels over time. (F) Swelling of MA-functionalized 15 wt % ChMA hydrogels (DM: 3.1%) over time.

physiological conditions.[21−23] Hydrogels based on HAMA have been extensively investigated for stability, and no chemical degradation under physiological conditions was found.[7,10,14,24] Here, the MA esters present in polymeric networks of CS(G)MA polysaccharides are shown to be susceptible to chemical degradation under physiological conditions. The mechanism for this degradation is explored, and the reasons for the differences in hydrolysis rates of MA esters present in HAMA and CS(G)MA before and after polymerization are elucidated.

2. RESULTS AND DISCUSSION

2.1. Derivatization and Characterization of Methacrylated Glycosaminoglycans. To systematically study MA ester hydrolytic (in)stability, HA, CS, and (desulfated) chondroitin (Ch) were derivatized with methacrylating agents to yield polysaccharides with incorporated GlyMA ester (R_1, as shown in Figure 1) or MA ester (R_2, as shown in Figure 1) groups.

First, CS (>60% sulfated at the 4-position, Sigma-Aldrich) was reacted with glycidyl MA in dimethyl sulfoxide (DMSO) to form CS (glycerol) methacrylate (CSGMA). The reaction was performed at multiple CS/glycidyl MA ratios to yield CSGMA with degrees of methacrylation (DM, defined as the number of MA and GlyMA groups per 100 disaccharide units and expressed as a percentage) of 3.4, 7.2, 14.7, and 27.2% [Table S2, both GlyMA and MA were measured via high-performance liquid chromatography (HPLC)].[5] This methacrylation followed both ring opening and transesterification mechanisms (Figure S3), with ∼65% of the MAs incorporated into the polymer being GlyMA and ∼35% MA-esters. This ratio was independent of the degree of MA substitution.

To make control hydrogels, chondroitin (Ch), CS, and HA functionalized solely with MA-ester moieties were synthesized. Functionalization was carried out by reaction with methacrylic anhydride in H_2O/dimethylformamide (DMF) to yield chondroitin methacrylate (ChMA, Figure 1B), CSMA (Figure 2C), and HAMA (Figure 2D) with DMs of 3.2, 6.1, 12.7, and

https://doi.org/10.1021/acsomega.1c03395
ACS Omega 2021, 6, 26302−26310

34.1%, respectively.[13] Methacrylation of CS or HA (M_w: 19.9 and 57.0 kDa, respectively) did not result in changes of M_w, as evidenced by GPC analysis, demonstrating that the methacrylation reactions did not result in chain scission. Further details of the analysis and methods regarding these syntheses are described in the Supporting Information.

2.2. Determination of Methacrylate Hydrolysis Rates from Soluble HAMA and CSGMA Polysaccharides. The hydrolytic instability of MA esters attached to CSGMA and HAMA was investigated. Specifically, methacrylated polysaccharides of sufficiently high DM were selected to allow detection of the hydrolyzed product at any timepoint (DM of 34.1 and 27.2% for HAMA and CSGMA, respectively). Since no effect of the DM on the MA ester hydrolysis rate was reported previously for methacrylated dextrans over a wide range of DM's, we did not investigate other DM's in this study.[23] Both methacrylated polysaccharides (HAMA and CSGMA) dissolved in buffer (50 mM phosphate, pH: 7.4, ionic strength: 154 mM) were incubated at 37 °C. The released GlyMA and MA over time were determined via HPLC, and the remaining polymer-attached MA esters were quantified using ^{1}H NMR (Figure 2A). For HAMA (DM = 34.1%), after 2 weeks, 41.1% ± 0.4% of the MA-esters were hydrolyzed. Correspondingly, 62.0% ± 3.1% of MA-esters were still attached to HAMA (Figure 2A). A difference of ∼3% in MA hydrolysis measurements is found, which is within the experimental error of the ^{1}H NMR analytical method. For CSGMA, both GlyMA and MA were detected with HPLC (Figure 2B). Again, good correlation between the MAs released and the remaining polymer-bound MAs over time was found (Figure 2B). 65% of the MA moieties attached to CS was found to be GlyMA-based, showing that ring opening was the favored methacrylation mechanism (Figure 2C).

The observed ester hydrolysis rate was calculated from the slope of a plot of the natural logarithm of the residual MA fractions versus time. All hydrolysis reactions were assumed to follow first-order reaction kinetics.[23] Evaluation of CSGMA-ester hydrolysis kinetics showed faster hydrolysis of the ester of CS's COOH and GlyMA when compared with the hydrolysis of the ester that connects the COOH of MA and CS (Figure 2C, orange line). Specifically, the observed GlyMA and MA-ester hydrolysis rates were 3×10^{-6} and 4×10^{-7} s^{-1}, respectively. Hydrolysis of all MA-ester bonds took 30 days (Figure 2C, light blue line). The observed rates of MA-ester hydrolysis from HAMA was calculated to be 3×10^{-7} s^{-1}. The equal MA hydrolysis rates indicate that hydrolysis of ester bonds that connect MA to the OH groups of CS and HA have equal hydrolytic sensitivity (Figure 2D). GlyMA incubated at 37 °C in a pH 11.0 solution was found to release only a minor amount of free MA after 4 days (Figure S4C), demonstrating that, surprisingly, the GlyMA-ester was stable under the experimental conditions.

2.3. Cross-Linked HAMA, CSGMA, CSMA, and Chondroitin Methacrylate Hydrogel Stability in Buffer. The stability of cross-linked hydrogels formed with CSGMA and CSMA was investigated by incubating in buffers of different pH values. An increase of hydrogel weight over time due to swelling was observed when CSGMA hydrogels were incubated in buffers of neutral or basic pH (Figure 3A). This swelling increased the hydrogel weight over time until the hydrogels lost shape fidelity and completely liquefied. In contrast, CSGMA hydrogels in a buffer of acidic pH (5.0) showed only initial swelling with no further increase in weight found over 50 days (Figure 3A). The swelling and dissolution observed in physiological buffer is indicative of a degradation mechanism where the cross-links between CSGMA chains are hydrolyzed over time.[11,22] To explain, the increased hydrogel swelling is due to hydrolysis of the ester bonds in the cross-linked network resulting in a decrease in network density, in combination with electrostatic repulsions between the anionic CSGMA chains.[25] By increasing the DM of the CSGMA used to make the hydrogel, the degradation time increases (Figure 3B). Specifically, CSGMA hydrogels prepared using a CSGMA DM of 3.4, 7.2, 14.7, and 27.2%, respectively, took <2, 9, 19, and 25 days until hydrogel dissolution when incubated at pH 7.4 and 37 °C. Similarly, increasing the buffer pH reduced the time until dissolution. For example, CSGMA hydrogels with a DM of 27.2% incubated at 37 °C and pH 8.0, 7.4, or 7.0, respectively, took 7, 25, and 49 days to liquefy. Considering that increasing buffer pH and decreasing CSGMA DM reduce the time until full hydrogel dissolution, CSGMA hydrogel degradation due to alkaline hydrolysis of the MA ester bonds is a likely mechanism. Most CSGMA hydrogels were stable in pH 5.0, except for the hydrogel based on CSGMA with a DM of 3.4%, which took 53 days to fully degrade (orange downward triangle, as shown in Figure 3B) due to uncatalyzed hydrolysis.

Swelling studies of hydrogels made from CSMA (containing only the MA moiety) were performed as a control experiment. CSMA-based hydrogels showed similar degradation in physiological buffer (pH 7.4, Figure 3C) as the CSGMA hydrogels shown in Figure 3A,B, indicating that both MA and GlyMA moieties are hydrolysis sensitive when attached to CS. Considering CS(G)MA hydrogels contain significant numbers of MA-based cross-links (at least up to 35% of the total number of MAs, see the section determination of methacrylate hydrolysis rates from soluble HAMA and CSGMA polysaccharides), it can be deduced that cross-links based on GlyMA and MA-esters both hydrolyze in a pH-dependent fashion. Specifically, CSMA (DM: 6.2%) and CSGMA (DM: 7.2) degraded in ∼7 and 8−9 days, respectively, when incubated at pH 7.4 and 37 °C. The similarity in degradation times between hydrogels of similar DM based on CSMA and CSGMA might indicate that cross-linking reduces the hydrolysis rate of GlyMA-esters and can prolong the time until full liquefaction for low DM hydrogels. To explain, in CSGMA hydrogels, the minimum number of cross-links required to maintain the hydrogel coherence depends on cross-links of GlyMA-esters and MA-esters. Interestingly, the timescale of a high DM CSGMA hydrogel degradation (25 days for CSGMA DM: 27.2% at pH 7.4 and 37 °C) is similar to the MA-ester hydrolysis rate of soluble CSGMA of the same DM incubated under the same conditions (Figure 2B). The CSGMA polymer retained ∼3% of attached MA groups after 25 days. These remaining MAs correspond to a CSGMA DM of ∼0.8% and solely consist of MA-esters (all GlyMA esters were hydrolyzed after 14 days). This similarity in degradation times between soluble polymer and polymer network indicates that cross-linking CSGMA into a network does not significantly change the hydrolytic sensitivity of the MA-ester bonds.

To confirm that CS(G)MA hydrogels degrade due to hydrolysis of the MA ester bonds, polymer M_w analysis was performed. Specifically, a liquefied sample of CSGMA hydrogel (DM: 14.7%, incubated in a 37 °C, pH 7.4 buffer for 20 days) was analyzed using GPC and compared to unmodified CS and CSGMA of the same DM (Figure 3D). The molecular weight

https://doi.org/10.1021/acsomega.1c03395
ACS Omega 2021, 6, 26302−26310

of the main peak of the chain distribution (M_P) of CS, CSGMA, and the soluble polymer chains obtained after degradation of CSGMA hydrogel was 19.8, 21.9, and 19.9 kDa, respectively. The liquefied hydrogel sample further showed a second peak shouldering the main peak with a peak molecular weight of 57.5 kDa (Figure S5), which can likely be ascribed to the presence of remaining CS polymer chains connected via an intact cross-link. Notably, the presence of the CS polymer in the liquefied hydrogel sample further corroborates that the mechanism of CSGMA hydrogel degradation is based on hydrolysis of the ester linkages and not by hydrolysis of glycosidic bonds in the main CS chain. The main degradation products of CSGMA, as measured via HPLC (see Figure S4C), were CS, MA, and GlyMA. The intermediate ester connecting the glycerol and MA groups in GlyMA was found to be more resistant to alkaline hydrolysis than the ester connecting GlyMA and CS (see Figure S4C and the related text), indicating that the ester bond between CS and GlyMA is hydrolyzed in the studied CSGMA hydrogel degradation. Proposed reaction mechanisms for the alkaline hydrolysis of both GlyMA and MA-ester linkages from CSGMA are presented in Figure S6.

In contrast to CS(G)MA hydrogels, cross-linked HAMA hydrogels (DM: 12.7%, derivatized with methacrylic anhydride) incubated in 37 °C at pH 5.0 to 7.4 did not show any signs of degradation over a 50 day period (Figure 3E). Previous studies on HAMA hydrogels functionalized with glycidyl MA or methacrylic anhydride also did not show any hydrogel degradation in physiological buffers.[7,14] One of the main structural differences between HA(MA) and CS(MA) is found in the sulfation. This sulfation can lead to increased hydration of CS over HA or extra inductive effects on the CS alcohol groups, both of which can lead to increased hydrolytic sensitivity.

To understand the effects of the sulfation of CS on ester hydrolysis in cross-linked CS(G)MA hydrogels, desulfated ChMA hydrogels were tested. First, CS was desulfated in acidic methanol to yield Ch polymers (see the Supporting Information). Ch was then derivatized with methacrylic anhydride to yield ChMA (DM: 3.1%). ChMA hydrogels were subsequently prepared and incubated in buffers of various pHs at 37 °C (Figure 3F). ChMA hydrogels incubated at pH 5.0 were found to be stable for >7 days, whereas the hydrogels incubated at pH 7.0−8.0 liquefied in less than 1 day in a pH-dependent manner. Specifically, ChMA hydrogels were found to be fully dissolved after 3, 5, and 7 h of incubation in pH 8.0, 7.4, and 7.0, respectively. The pH-dependent degradation of ChMA hydrogels indicates that desulfated ChMA hydrogels are subject to the same degradation as CS(G)MA hydrogels and that the sulfate moieties present in CS(G)MA are not mainly responsible for CS(G)MA MA ester liability to hydroxide-mediated hydrolysis.

No publications examined the swelling characteristics over time of hydrogels solely based on CS(G)MA. However, swelling studies were reported on hydrogels based on CS(G)MA combined with other polymers like polyethylene glycol diacrylate.[8,9,26,27] The hydrolytic stability of other MA ester-coupled polysaccharides as soluble polysaccharides and polymeric networks has previously been investigated.[22,23] MA-esters derivatized onto dextrans were previously found to only degrade as free polysaccharides but not in their cross-linked states. This difference in ester stability is attributed to the hydrophobic groups present in the polymerized MA backbone of the cross-linked network. These groups results in a more hydrophobic microenvironment around the ester groups protecting them against nucleophilic attack.[23,28] The difference in MA ester stability between soluble HAMA and HAMA networks is thus in line with what was observed for methacrylated dextrans. Although no difference in the ester hydrolysis rate between soluble HAMA and CS(G)MA was found, a large difference in stability is observed when these polysaccharides were converted into cross-linked networks as HAMA hydrogels did not degrade, whereas CS(G)MA hydrogels experienced degradation due to hydroxide-driven hydrolysis.

An explanation for this difference in hydrolytic stability can be found in the molecular conformations of HA and CS. Specifically, hydrogen bonds that are formed between different groups of the repeating disaccharides are known to influence the rigidity of polymers, which in turn can influence the hydrolytic stability of groups on these polymers. For instance, in a study by Satelle et al., 2D NMR analysis of oligosaccharides of HA, 4-sulfated CS, and Ch showed that the equatorial 4-hydroxyl group (circled in orange, as shown in Figure 4A) in HA has a high propensity to form intramolecular

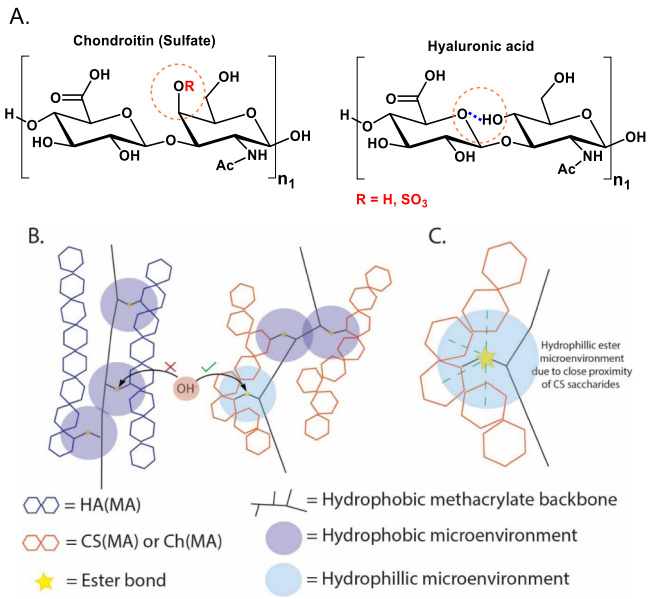

Figure 4. (A) Chirality at the 4-position of the amino sugar is different between Ch/CS and HA (circled in orange); for CS/Ch, this group is axial, for HA it is equatorial, allowing an intrapolymer hydrogen bond (blue line) formation with the neighboring 5-oxygen in the glucuronic acid ring. (B,C) Lack of intramolecular hydrogen bonds allows for higher polymer flexibility and hydrophilicity in CS(G)MA, causing the formation of more hydrophilic areas around the polymerized MA esters. These areas make the ester bonds more susceptible to nucleophilic attack by hydroxyl ions and subsequent hydrolysis.

hydrogen bonds (blue dashed line, as shown in Figure 4A) with the oxygen atom at the 5-position present in glucuronic acid, effectively creating a hydrogen bond that spans over the (1 → 3) linkage in HA. The axial 4-hydroxyl or 4-sulfate present in Ch or 4-sulfated CS (circled in orange, R═H, and R═SO_3^-, as shown in Figure 4A, respectively) was found not to be able to form the same hydrogen bond.[29] The presence/absence of this hydrogen bond for HA/CS/Ch is corroborated by another NMR study carried out on methylated and

https://doi.org/10.1021/acsomega.1c03395
ACS Omega 2021, 6, 26302−26310

unmethylated HA/CS dissolved in dimethyl sulphoxide-d_6 by Heatley et al.[30] Specifically, based on a series of measurements at different temperatures and concentrations, it was found that the 4-hydroxyl group of the hexosamine is hydrogen bonded to the oxygen in the 5-position on the uronic acid residue for HA. No similar temperature/concentration-dependent hydrogen bond over the (1 → 3) linkage in CS was found. In X-ray crystallography studies performed by Winter et al. on HA and CS, intrapolymer hydrogen bonds for both HA and CS (4-sulfated) were found to span over the polysaccharide's (1 → 4) disaccharide linkage.[31,32] For HA, the intrapolymer hydrogen bond over the (1 → 3) linkage was also verified. In contrast, for CS, no intrapolymer bond was found to occur between the 4-sulfate and the uronic acid ring oxygen groups, further verifying the difference in intrapolymer hydrogen bonding for HA and CS/Ch.

In addition to NMR and X-ray crystallography, infrared spectroscopy has also been used to characterize the structure of HA and CS. Specifically, in a FT-IR study of HA, a high degree of rigidity along the polysaccharide chain was found.[33] This high order along the length of the HA chain was determined to be due to the intrapolymer bonds found to be present for HA over the (1 → 3) and (1 → 4) glycosidic linkages, which hamper rotation around these linkages. The intrapolymer hydrogen bond found between the amide and carboxylate of HA was not found to influence polysaccharide rigidity at neutral pH.[34] FT-IR studies of CS mentioned no specific intrapolymer hydrogen bonds. This is possibly due to the additional technical difficulty that the randomly sulfated CS polysaccharide poses in resolving the molecular conformation using infrared-based techniques.[35]

The proposed dependence of hydrolysis on a change in polysaccharide rigidity due to chirality is further corroborated by a combined spectroscopy and molecular dynamics study from Pichert et al. In this study, the equatorial position of the hydroxyl group in HA was shown to lead to an increase in intrapolymer hydrogen bonds and less available energetically favorable conformational states when compared with CS/Ch. Specifically, HA formed 1.6× more hydrogen bonds than Ch due to this intramolecular bonding, whereas Ch/CS was able to form more hydrogen bonds with surrounding water molecules, increasing its hydration.[36] Furthermore, a recent study combining 2D-IR, molecular dynamics, and single-chain force microscopy measurements on HA shows that HA's chain rigidity scales with the strength of its intrapolymer hydrogen bond network.[37,38]

All the combined reported findings suggest that Ch and CS are more hydrated and more flexible as compared to HA due to a change in the intrapolymer hydrogen bond spanning over the (1 → 3) linkage in HA.

When relating the previous findings on HA/CS/Ch intramolecular bonding to the hydrolytic stability of cross-linked HAMA networks over CS(G)MA networks, the effect of methacrylation on HA/CS/Ch molecular conformations should be taken into account. The MA esters studied here are installed on either the carboxylic acid or primary alcohol groups of HA/CS/Ch. Practically, the MAs are not spatially near any of the groups involved in the HA intrapolymer hydrogen bond over the (1 → 3) linkage (see e.g. Figure 1). The polysaccharides studied here had 3.4−34.1% of their disaccharide repeating units methacrylated, leaving between 96.6 and 65.9% of the repeating disaccharides unmodified, meaning large numbers of repeating units on the polysaccharides were in the same state as can be found in native HA/CS/Ch. In addition, increasing the polysaccharide DM did not hamper hydrolysis in CSGMA hydrogels, indicating that any potential influence of the MA groups on the effective conformation of the polysaccharides, especially the intrapolymer hydrogen bond present in HA(MA), can be considered minimal when regarding the MA ester hydrolysis studied here.

It is likely that the esters in CS(G)MA hydrogels are less protected by the local hydrophobic environment of the MA backbone formed during cross-linking due to the inherently higher hydrophilicity and conformational flexibility of the CS/Ch backbone. The influence of the sulfates on CS(G)MA-ester hydrolysis is limited, considering that the rate of MA-ester hydrolysis from polymeric CS(G)MA and HAMA was similar, and that alkaline hydrolysis also occurred in desulfated ChMA hydrogels. Therefore, the greater hydrophilicity and conformational flexibility of CS over HA due to the difference in chirality of the amino sugar explain the hydrolytic (in)stability of the ester bonds in cross-linked HAMA and CS(G)MA.

3. CONCLUSIONS

To conclude, cross-linked hydrogels based on CS(G)MA degrade in physiological buffer and 37 °C by hydrolysis, whereas HAMA-based hydrogels are stable under the same conditions. The flexible and hydrophilic nature of CS chains results in a hydrophilic microenvironment around the ester bonds connecting CS to the polymerized MA backbone. This local microenvironment allows for nucleophilic attack of hydroxyl ions on the carbonyl carbon of the esters, leading to hydrolysis of the MA ester bond and CS(G)MA hydrogel degradation. This study reports on the effects of monomer chirality on hydrogel degradation which is highly relevant for biomaterial scientists developing biocompatible and/or degradable hydrogels. Based on this study, hydrogels based on CS(G)MA can potentially serve as hydrolytically degradable implants for use in tissue engineering and pharmaceutical applications.

4. MATERIALS AND METHODS

4.1. Chemicals. All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) and were used as received. All solvents were acquired from Biosolve (Valkenswaard, the Netherlands). Sodium HA (M_w of 57.0 kDa) was obtained from Lifecore Biomedical (Chaska, MN, USA). CS was extracted from bovine trachea and contained at least 60% 4-sulfated polysaccharide (Sigma-Aldrich). To simplify illustrations, only the main sulfated monomer of CS (chondroitin-4-sulfate) is drawn in the figures. All buffers used were supplemented with 0.02 wt % sodium azide to prevent bacterial growth. All buffers had a concentration of 50 mM and were supplemented with sodium chloride up to physiological ionic strength. For pH 5.0, sodium acetate was used and for the pH range 7.0−8.0, sodium phosphate buffers were used. Glycidyl MA was purchased form Sigma-Aldrich, and reference GlyMA was synthesized according to the study by Ratcliffe et al.[39]

4.2. Desulfation of Chondroitin Sulfate. CS was desulfated to yield chondroitin via acidic methanolysis, as clarified in Scheme S1. CS (5 mg/mL, for a total of 25 g of CS) was dispersed in methanol supplemented with 0.5 wt % acetyl chloride under vigorous stirring for 7 days.[40] The acidic

https://doi.org/10.1021/acsomega.1c03395
ACS Omega 2021, 6, 26302−26310

methanol was replaced on days 1 and 3. Removal of the acetic methanol was carried out by centrifugation (1000*g* for 15 min) of the dispersion to allow the CS to settle, followed by decantation of the methanol followed by drying under gentle N_2 flow.

The product, methylated chondroitin (methyl-Ch), was dissolved in deionized water supplemented with 0.5 M NaCl (50 mg of the initial CS per mL) and precipitated in cold ethanol (−20 °C) to remove excess methanol. After decantation of the ethanol, the product was dried under a gentle flow of N_2. Subsequently, methyl-Ch was dissolved in 0.1 M NaOH solution (25 mg/mL) and stirred for 24 h. The basic demethylated chondroitin solution obtained was then neutralized using acetic acid, supplemented with NaCl up to a concentration of 0.5 M, and precipitated in cold ethanol, as described above. The collected chondroitin was again dissolved in water and dialyzed for 2 days against water (a dialysis membrane with a molecular weight cut-off of 14 kDa). The dialyzed chondroitin solution was then freeze dried to yield chondroitin as an off-white powder and characterized using elemental analysis (see Table. S1, service provided by MikroLab Kolbe, Oberhausen, Germany).

4.3. Synthesis of Methacrylate-Functionalized Hyaluronic Acid, Chondroitin, and Chondroitin Sulfate. Methacrylated derivatives of HA, CS, and chondroitin (Ch) were synthesized. The DM is defined as the number of MA groups per 100 disaccharide units and expressed as a percentage. Two separate methacrylating agents were used to synthesize methacrylated CS(G)MA with different MA moieties, namely, glycidyl MA and methacrylic anhydride. The feed ratios of methacrylating agents to polysaccharide were calculated per mole of HA/CS/Ch monomeric unit.

4.3.1. Synthesis of CSGMA Functionalized with Glycidyl Methacrylate. For the methacrylation of CS with glycidyl MA, a previously published method was followed in which first the lipophilic *tert*-butyl-ammonium salt of CS was formed, after which the methacrylation was performed in DMSO as a solvent for 2 day at 50 °C.[5] Molar feed ratios of glycidyl MA to CS-TBA disaccharide of 0.2, 0.4, 0.6, and 1:1 were used to produce CSGMA with increasing DM. For an extended analysis of the resulting CSGMA using NMR and HPLC techniques, see the text accompanying Figures S3 and S4.

4.3.2. Synthesis of HAMA, CSMA, and Chondroitin Methacrylate Functionalized with Methacrylic Anhydride. A method for synthesis of HAMA functionalized with methacrylic anhydride in a water/DMF solution adapted by Abbadessa et al.[6] was used to synthesize HAMA, CSMA, and chondroitin MA of various DM. For HAMA, methacrylic anhydride was added in molar feed ratios of 2.2:1 (anhydride/HA disaccharide). For the synthesis of CSMA and ChMA, the same procedure and concentrations as for the synthesis of HAMA were performed, with a molar feed ratio of 3:1 (anhydride/CS disaccharide or anhydride/Ch disaccharide) for both reactions. The DMs of the obtained HAMA, CSMA, and ChMA were determined using the HPLC method described below.

4.4. HAMA, CS(G)MA, and Chondroitin Methacrylate Polysaccharide Characterization. *4.4.1. Degree of Methacrylation Determinations Using HPLC.* Sample preparation was performed by dissolving 15 mg of dry, accurately weighed polysaccharide in 5 mL of 0.02 M NaOH solution overnight at 37 °C to ensure full hydrolysis of both MA and GlyMA groups from the polysaccharide. Next, 1 mL of 2 M acetic acid was added to neutralize the solution.[41]

MA and GlyMA concentrations in the different samples were determined with reversed phase HPLC with an Alliance Waters HPLC system equipped with UV−vis detection (Dual Lambda absorbance, monitoring at 210 nm). Samples of 10 μL were injected and passed through a Waters Sunfire C18 column (flow rate: 1 mL/min, heated at 50 °C). An isocratic method was used where the eluent consisted of 3:97 acetonitrile/Milli-Q water (v/v) with a pH adjusted to 2 (perchloric acid). Each injection of solutions that contained CS(G)MA, CS, chondroitin, or ChMA was followed by flushing the column with the eluent for 30 min to clean the column. The MA and GlyMA contents of the samples were quantified using a calibration curve of both compounds. From the quantified released moles of MA and GlyMA per sample, the DM of the initial polysaccharide was calculated. In addition, this method was used to check the MA conversion after photopolymerization of cross-linked hydrogels by using 15 mg of freeze-dried hydrogel instead of polysaccharide.

4.4.2. 2D NMR Analysis of CS(G)MA. HSQC ^{1}H and ^{13}C spectra of CS and CS(G)MA in D_2O were recorded using an Agilent-400 MHz spectrometer. Spectra were analyzed using Mestrenova software. All shown spectra were calibrated according to the position of the CH_2 found in unsulfated-CS on the 6 position of the *N*-acetyl-galactosamine (3.45 by 61 ppm, ^{1}H and ^{13}C signals, respectively).

4.4.3. Molecular Weight Determinations of (Methacrylated) Polysaccharides Using GPC. For determination of the molecular weights of the polysaccharides, a GPC method was used. Samples of polysaccharide (5 mg/mL) were dissolved in the eluent consisting of 0.3 M ammonium acetate (pH 5.0, set with 1 M HCl) or in pH 7.4, 50 mM sodium phosphate buffer overnight. Then, samples were injected (50 μL) into an Alliance Waters HPLC system equipped with a RI detector and a double PL aquagel OH-mixed column (M_w range: 0−300 kDa) with the attached guard column to remove potential particulates. The column was kept at a temperature of 40 °C, and the flow rate was 1 mL/min. The runtime of was 30 min. Calibration was carried out using HA of different molecular weights (from 0.466 to 132.0 kDa). The M_w and M_n of these HA standards were provided by Lifecore Biomedical (Chaska, MN, USA) and measured using GPC-MALLS (multiangular laser light scattering, as performed by the supplier) (see the table in Figure S2 for an overview of used HA standards).

4.5. Kinetics of Methacrylate Hydrolysis of Soluble HAMA and CS(G)MA. HAMA and CSGMA were separately dissolved in buffer (pH 7.4) at a concentration of 5 mg/mL. Subsequently, these solutions were incubated at 37 °C up to 30 days. At different timepoints, samples were drawn and acidified using 0.5 mL acetic acid to stop further hydrolysis. Half of each sample solution was used to measure the GlyMA and MA contents, while the other half was transferred into dialysis cassettes (molecular weight cut-off: 2 kDa) and dialyzed for 4 days against water (RT, pH 5−6) to remove GlyMA and MA. Next, the solutions were freeze dried to yield a dry white powder. The freeze-dried polysaccharides were dissolved in D_2O and analyzed using ^{1}H NMR. From the spectra obtained, the amount of MA groups bound to HAMA or CS(G)MA was calculated, as described previously.[5,6,13]

4.6. Hydrogel Experiments. *4.6.1. Fabrication of Hydrogel Discs.* Polysaccharide solutions consisting of 15 wt % of either CSGMA, HAMA, ChMA, or CSMA in milliQ

https://doi.org/10.1021/acsomega.1c03395
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water were prepared by dissolving the polysaccharides overnight at 4 °C on a roller bench. Before exposure to UV-irradiation, the solutions were supplemented with 0.1 wt % Irgacure 2959 photoinitiator (IG2959, Sigma-Aldrich), vortexed for 10 s, and centrifuged (1000*g* for 5 min) to remove air bubbles. Cross-linked hydrogel disks were made by injecting the polysaccharide solution into a Teflon mold with cylindrical wells (height: 2 mm, circular diameter: 6 mm) capped with a quartz glass plate. Next, the polysaccharide solutions were UV-irradiated for 10 min at a distance of 5 cm of a point light source UV lamp to yield photopolymerized hydrogel discs (Bluepoint 4 UV lamp, Honle UV technology AG, intensity at 5 cm: 103 mW/cm^2, wavelength range: 300−600 nm). MA conversion after UV polymerization was determined via HPLC and was >95% for all hydrogels.[41]

4.6.2. Hydrogel Degradation Studies. Cross-linked hydrogel discs were placed in pre-weighed glass vials, and their starting weights (W_0) were determined. Buffers of varying compositions (pH ranged from 5.0 to 8.0, always 1 mL in volume) were then pipetted into each vial, and samples were incubated at 37 °C. At various timepoints, the buffer solution was replaced, and the weight of hydrogel determined (W_t). The weight ratio (equal to W_t/W_0) was then calculated for every timepoint.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03395.

Data on the (and mechanism of) desulfation of CS to Ch, GPC analysis of polysaccharides, determination of polysaccharide MA ester and GlyMA ester methacrylation grades, extended analysis of the MAs esterified onto CS(G)MA (2D-NMR, HPLC, and proposed reaction mechanism), and the proposed reaction mechanisms for the ester hydrolysis of MA- and GlyMA-esters from CS (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

C.C.L.S. acknowledges personal funding from the Future Medicines Program financed by the Netherlands Organisation for Scientific Research (NWO no. 022.006.003).

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