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Terminal-end functionalization of chondroitin sulfate for the synthesis of biomimetic proteoglycans

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ABSTRACT

Chondroitin sulfate (CS) based bottle brush proteoglycan mimetics may be employed to restore tissue functionality. Synthesis of CS bottle brush structures requires immobilization of CS at its terminal end. In this study, we investigated commercially available natural CS for use in CS bottle brush synthesis. A terminal primary amine on CS was identified and utilized to conjugate amine-reactive vinyl monomers (*i.e.* acrylic acid and allyl glycidyl ether). Conjugation of vinyl monomers to the CS terminal amine was confirmed using a fluorescamine assay, ^{1}H NMR, and ATR-FTIR. CS was also immobilized onto epoxy functionalized surfaces *via* the CS terminal primary amine as confirmed by contact angle measurements of surface wettability. Attachment of polymerizable end groups to CS and attachment of CS to functionalized substrates demonstrated here are the first steps towards synthesis of CS bottle brush PG mimics.

1. Introduction

Aggrecan, an aggregating proteoglycan, is a structurally and osmotically important proteoglycan in load bearing tissues such as articular cartilage and the nucleus pulposus of the intervertebral disc. Aggrecan consists of a protein core to which primarily chondroitin sulfate (CS) glycosaminoglycan chains are covalently attached in a dense array resulting in a bottle brush macromolecule. Aggrecan is lost from cartilage and the intervertebral disc with aging and degeneration and its loss is associated with mechanical deterioration of these tissues leading to the progression of osteoarthritis and intervertebral disc degeneration.

The fabrication of synthetic and natural based biomimetic bottle brush polymers is under considerable investigation (Grande, Baskaran, Baskaran, Gnanou, & Chaikof, 2000; Miura, 2007; Muthukrishnan et al., 2005; Sheiko, Sumerlin, & Matyjaszewski, 2008). Several bottle brush polymers have been formulated with mono- and disaccharide side chains (Miura, 2007; Muthukrishnan et al., 2005). These glycopolymers have been demonstrated to have biological function; however their use as mechanically stabilizing materials has not been investigated due to their short sugar side chains. Glycopolymers can be synthesized *via* "grafting-to" or "grafting-through" techniques. In the "grafting-to" technique glycopolymers are formed by the chemical modification of preformed polymers or polymeric substrates with sugar-containing reagents. Although this technique is straightforward, it often results in less regular structures with limited glycosylation. In the "grafting-through" technique, sugar-bearing monomers are polymerized into linear polymers with pendant sugar moieties. The majority of glycopolymers have been synthesized by the polymerization of sugar-containing vinyl monomer however the preparation of the polymerizable sugar-monomers is often a complex and multi-step process that requires chemical modification of the sugar moiety. Chondroitin sulfate has many potential therapeutic benefits and the structure of CS is strongly linked to its function however, natural CS has yet to be synthesized into a bottle brush polymeric structure for therapeutic applications (Malavaki, Mizumoto, Karamanos, & Sugahara, 2008; Yamada & Sugahara, 2008).

In order to immobilize CS in biomimetic brush structures, long CS chains must be immobilized by their terminal end to a linear polymeric backbone, requiring unique terminal end chemistry on the CS chain. CS is rich in functional groups including primary

Abbreviations: PGs, proteoglycans; GAG, glycosaminoglycan; CS, chondroitin sulfate; PA, primary amines; BSA, bovine serum albumin; GalNAc, *N*-acetyl-D-galactosamine; GlcUA, glucuronic acid; AA, acrylic acid; AGE, allyl glycidyl ether; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; sulfo-NHS, sulfo-NHS (*N*-hydroxysulfosuccinimide); ^{1}H NMR, proton nuclear magnetic resonance; ATR-FTIR, attenuated total reflectance-Fourier transform infrared spectroscopy; CPC, cetylpyridinium chloride.

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and secondary alcohols, carboxylic acids, amides, and sulfate groups throughout the CS backbone however a unique terminal group must be utilized in the synthesis of biomimetic brush macromolecules.

All glycosaminoglycans, with the exception of HA, are synthesized as proteoglycans, *i.e*. with a covalent linkage to a protein (Kjellen & Lindahl, 1991). CS is linked to the aggrecan core protein at its L-serine residue where a glycosidic bond is formed between the linkage CS saccharide xylose and the hydroxyl group of serine (Sugahara, Ohi, Harada, De Waard, & Vliegenthart, 1992; Sugahara, Yamashina, De Waard, Van Halbeek, & Vliegenthart, 1988; Vertel, 1995). In the isolation of CS, the proteoglycan core protein can be broken down by either enzymatic digestion or alkaline treatment releasing the GAG chains and leaving the CS chain with its linkage region intact and still connected to protein residue (Anderson, Hoffman, & Meyer, 1965; Mattern & Deen, 2007; Volpi, 2006). The resulting terminal protein residue on the CS chain has been utilized to immobilize CS onto agarose membranes using the 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate (CDAP) activating agent to form an isourea bond (Mattern & Deen, 2007). CS isolated from rat chondrosarcoma cell cultures and digested with proteinase K, to maintain the terminal amino acid residue, was also utilized to end-graft CS by first reacting the terminal reactive amine group with dithiobis sulfosuccinimidyl propionate (DTSSP) then reducing it to a thiol group for coupling to gold substrates (Seog et al., 2002, 2005). These studies demonstrate the utility of the CS-terminal amino residue.

Commercially available CS however is inhomogeneous in its physical and chemical properties. Depending on the isolation and purification techniques used, CS has variable structure, properties, purity, molecular weight and molecule integrity (Lauder, 2009; Volpi, 2006). Parameters reported in association with CS production are limited and mainly consist of elementary chemical composition, impurities and general ranges of MW (Volpi, 2007). No information is provided on the terminal end of CS or if the linkage region of CS has remained intact after the isolation procedure and these properties are not generally investigated by manufacturers.

In this work, we investigated the end functionalization of CS for subsequent fabrication of CS brush structures utilizing the terminal protein residue of commercially available CS where, immobilization of CS *via* its terminal end is critical for the fabrication of brush structures. The studies described here demonstrate a facile route to the end functionalization and immobilization of commercially available CS.

2. Materials and methods

2.1. Materials

Chondroitin-4-sulfate, chondroitin-6-sulfate and mixed CS powders were purchased from Sigma–Aldrich and Calbiochem and originated from different animals and tissues. L-Serine, bovine serum albumin and *N*-acetyl-D-galactosamine were purchased from Sigma–Aldrich and used as control samples. Fluorescamine and all other reagents were purchased from Sigma–Aldrich and used as received.

2.2. Determination of CS primary amine content

A fluorescamine assay was developed and validated for use in the determination of the primary amine (PA) content of CS. Fluorescamine, a fluorometric reagent, reacts directly with primary amines to yield highly fluorescent derivatives (390 nm excitation, 475 nm emission) whose resulting fluorescence is proportional to the amine concentration (Udenfriend et al., 1972). Reactions of fluorescamine with primary amines proceed at room temperature with a half-time of a fraction of a second while excess reagent is destroyed with a half-time of several seconds (Held, 2001). The fluorescamine reagent is particularly suitable for the detection of a single PA in CS as it is sensitive to PAs in the picomolar range (Udenfriend et al., 1972).

In a general procedure, 150 μl of sample solution is plated in triplicate in a flat bottom black 96-well plate. Fluorescamine solution is prepared at 3 mg/ml in DMSO. A 50 μl aliquot of the fluorescamine solution is added to the sample solutions then allowed to react for 5 min at room temperature with constant agitation on a shaker plate. Sample fluorescence was measured on an Infinite M200 TECAN spectrophotometer with excitation/emission of 365/490 nm. Sample free buffer solution with fluorescamine reagent was used as a blank for fluorescence readings.

Detection of primary amines with fluorescamine was calibrated with solutions of known amine content. L-Serine (MW, 105.09) which is the attachment site for CS to the aggrecan core protein, and contains only one PA per molecule, was used to establish a fluorescence *vs.* [PA] standard curve. The number of PA/molecule of a sample can be calculated from the linear region of the L-serine standard curve. Bovine serum albumin (BSA) (MW, 66,432 Da) (BSA) was used as a positive control biomolecule with a known high PA content and *N*-acetyl-D-galactosamine (MW, 221.2 Da) was used as a negative control as it is a secondary amide containing component of CS but contains no PAs. Samples were solubilized in sodium borate buffer (SBB, 0.1 M, pH 9.4) then diluted in a serial dilution and assayed with the fluorescamine reagent. Fluorescence was normalized to SBB blanks and samples were taken in triplicate. The number of primary amines per molecule was calculated at each concentration from the linear region of the L-serine standard curve.

CSs from various sources were assayed for their primary amine content using the validated fluorescamine assay. Chondroitin-4-sulfate sample MW was estimated to be 22 kDa while primarily chondroitin-6-sulfate samples were estimated to have a MW of 65 kDa based on literature values (Buzzega, Maccari, & Volpi, 2009; Volpi, 2007). Fluorescence was normalized to SBB blanks and samples were taken in triplicate.

2.3. Chondroitin sulfate–monomer conjugation

Two amine reactive chemistries were investigated for the conjugation of monomers to the CS chain at its terminal primary amine end. The monomers allyl glycidyl ether (AGE) and acrylic acid (AA) (Sigma–Aldrich) were used. AGE, is an epoxide and vinyl containing monomer, which will react with amines at alkaline pH (Hermanson, 2008). Acrylic acid is a polymerizable monomer with carboxylic acid functional groups. The carboxylic acid group of acrylic acid can be activated to be highly reactive with amines using well characterized EDC/sulfo-NHS coupling reactions (Hermanson, 2008).

For the conjugation of CS to AGE, solutions of monomer at various concentrations in 0.1 M SBB, pH 9.4, were mixed with solutions of CS in 0.1 M SBB pH 9.4 to achieve varying monomer:CS molar ratios. Acrylic acid was first activated with EDC/sulfo-NHS (2 mM EDC and 5 mM sulfo-NHS in MES buffer (0.05 M MES, 0.5 M NaCl), pH 6.0) for 15 min followed by quenching of excess EDC with 2-mercaptoethanol (10 min at a final concentration 20 mM). Activated acrylic acid was then combined with CS (CS in phosphate buffered solution, pH 7.4) at varying monomer:CS molar ratios. All CS-monomer solutions were placed on a rotator (Thermolyne Labquake) and allowed to mix. CS–acrylic acid solutions were then filtered using a sephadex G-25 pre-packed desalting column equilibrated in PBS (PD-10, GE Healthcare) to remove excess reactants. 150 μl samples of each solution were taken in triplicate and assayed with the fluorescamine assay for their PA content. The percentage of PAs in the CS sample conjugated to monomer is indicated by the

percent decrease in PA content which was calculated as:

$$\text{Percent conjugation} = 100\% \times \frac{([\text{PA}]\ \text{in CS without monomer} - [\text{PA}]\ \text{in CS with monomer})}{[\text{PA}]\ \text{in CS without monomer}}$$

A decrease in the PA content of the CS–monomer solutions with respect to CS without monomer is indicative of binding of the monomer to CS at the primary amine. Solutions of monomer without CS were also assayed with the fluorescamine reagent and found to exhibit no appreciable fluorescent signal at the excitation/emission of interest.

2.4. Proton nuclear magnetic resonance (^{1}H NMR) and Fourier transform infrared spectroscopy (FTIR) analysis of AGE–CS conjugation over time

Samples of CS–AGE conjugate prepared at the 1000:1 ratio were investigated with ^{1}H NMR to further demonstrate CS–monomer conjugation. Samples were prepared as described previously followed by extensive dialysis against DI water (regenerated cellulose membrane, MWCO 6–8 K) in order to remove unreacted monomer. Purified samples were lyophilized then reconstituted in D_2O at approximately 30 mg/ml. ^{1}H NMR spectra were taken on a 300 MHz NMR spectrometer (UNITYNOVA) at 64 scans and at ambient temperature. Lyophilized samples were also investigated on an attenuated total reflectance (ATR)-FTIR (Thermo Nicolet Nexus 870 FT-IR Spectrometer with a single bounce ZnSe crystal ATR accessory, 64 scans) for chemical composition and compared to that of natural CS.

2.5. Modification and characterization of glass surfaces with chondroitin sulfate

Epoxide functionalized glass slides (Genetix) were utilized for the immobilization of CS. Functionalized glass slides as well as unfunctionalized glass slides were cut into 1 cm × 1 cm samples, placed in 6-well plates and soaked in 3 ml of CS solution in SBB pH 9.4 at varying CS concentration. Well plates were placed on a shaker plate and samples were allowed to incubate with constant agitation for 4 h at room temperature. Slides were subsequently rinsed thoroughly (3× in SBB) then placed in 3 ml of SBB and allowed to rinse for an additional 3 h with constant agitation to remove any non-covalently bound CS. Slides were rinsed an additional three times in SBB then allowed to air dry. Unfunctionalized glass slides were similarly prepared as control samples.

CS modified glass slides were analyzed for their hydrophilicity using a contact angle goniometer with water as a medium. Three readings from triplicate samples of epoxide and unfunctionalized slides soaked in CS and buffer solutions were taken. A decrease in contact angle is indicative of an increase in surface hydrophilicity imparted by charged CS molecules covalently attached to the glass surfaces.

3. Results

3.1. Primary amine content of CS from various sources

In order to establish a relationship between fluorescamine fluorescence intensity and primary amine content of a CS sample, L-serine was used to establish a standard curve where L-serine is the amino acid residue of CS and contains one primary amine per molecule. Fluorescamine signal had a linear relationship with L-serine concentration to approximately 3500 fluorescence units corresponding to a L-serine concentration of 0.625 mM. Molarity

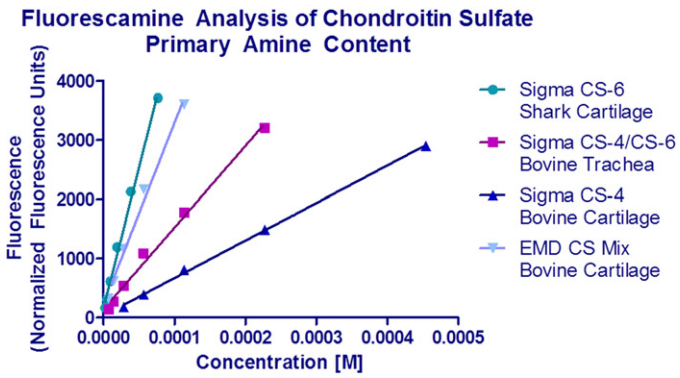


Fig. 1. Fluorescamine analysis of chondroitin samples from various sources.

of L-serine which corresponds to primary amine molar concentration [PA], in a one to one relationship, was plotted *vs.* fluorescence. The linear region of the fluorescence *vs.* [PA] curve was taken from 0.020 mM to 0.625 mM PA concentration. This corresponded to fluorescence intensities of 138 and 3419 normalized fluorescence units respectively. The linear relationship of:

$$[\text{PA}] = 5.73 \times 10^6 \times \text{fluorescence intensity}$$

where [PA] is the milimolar concentration of primary amines in the sample and fluorescence intensity is normalized to a blank ($r^2 = 0.98$). This linear relationship was used in order to determine the PA content of different biomolecule samples.

Bovine serum albumin (BSA) and *N*-acetyl-galactosamine (GalNAc) were investigated as positive and negative controls for PA concentration respectively. The protein BSA was used as a positive control and had a high PA content (19.74 ± 4.45 PA per molecule) as expected from the many PA containing amino acid residues in the BSA protein backbone. GalNAc had an average of 0.0 ± 0.05 PA per molecule indicating that the amide of GalNAc in the CS disaccharide does not contribute to the CS PA signal.

The presence of PA in CS from various sources and vendors was investigated using fluorescamine. A strong linear relationship (Fig. 1, $R^2 > 0.99$) between observed fluorescence and CS concentration for all CS samples indicates the fluorescamine assay as an appropriate means to measure small fractions of PA in the GAG samples. The number of PAs per chondroitin sulfate chain was calculated for each concentration of CS based on the L-serine standard curve and averaged. The average number of PAs per CS chain for the various types and sources of CS investigated are presented in Table 1. CS-6 from shark cartilage (Sigma) had the highest PA content of all chondroitin sulfate samples tested. CS from bovine trachea from both Calbiochem and Sigma had PA contents greater than one PA per CS chain while CS-4 from bovine cartilage supplied by Sigma had an average number of PAs per CS chain of 1.15 ± 0.7. Therefore, CS-4 C6737 from Sigma was investigated further for the application of CS to the synthesis of biomimetic aggrecan brush structures.

3.2. Chondroitin sulfate conjugation to synthetic monomers via the terminal primary amine

3.2.1. Fluorescamine analysis of CS–monomer conjugation

Two amine reactive chemistries were utilized to conjugate vinyl monomers to the CS terminal PA. The monomers allyl glycidyl ether (AGE) and acrylic acid were investigated. The AGE–CS and acrylic acid (AA)–CS reactions were investigated for concentration and temporal dependence. AGE Conjugation was dependent both on concentration and reaction time (Fig. 2 (top)). For the 1:1 AGE:CS molar ratio, no significant increase in percent conjugation is seen

Table 1
Estimated number of primary amines per molecule for chondroitin sulfate samples based on the L-serine fluorescamine standard curve.

Chondroitin sulfate	Tissue source	CS type	Estimated MW	Average # PA/CS chain	Standard deviation
Sigma C4384	Shark cartilage	Primarily CS-6	~65,000	10.52	1.26
Sigma C9819	Bovine trachea	60% CS-4, 40% CS-6	~22,000	3.08	0.38
Sigma C6737	Bovine cartilage	Primarily CS-4	~22,000	1.15	0.07
Calbiochem 230699	Bovine trachea	Mix of CS-4, CS-6, CS-4,6, CS-2,6	~22,000	7.16	0.95

Table 2
One-phase association curve fit parameters for AGE–CS reactions at varying AGE:CS ratios.

One-phase association: $Y = \text{plateau} \times (1 - \exp(-K \times x))$				
AGE:CS molar ratio	1:1	10:1	100:1	1000:1
Plateau (% conjugation)	12.5	58.8	81	96.3
K (1/h)	0.124	0.0187	0.203	1.32
Tau (h)	8.068	53.36	4.924	0.7575
95% confidence intervals ($n = 3$)				
Plateau (% conjugation)	7.1–17.8	32.4–85.3	76.6–85.4	93.7–98.9
K (1/h)	0.0–0.390	0.002–0.036	0.155–0.252	1.155–1.485
Tau (h)	2.563 to +infinity	28.09–531.1	3.977–6.464	0.674–0.866
R^2	0.399	0.814	0.930	0.936

after 4 h ($p > 0.05$) however for the higher ratios of 10:1 and 100:1 AGE:CS, percent conjugation significantly increased after up to 72 h of reaction ($p < 0.01$). For 1000:1 reactions, near complete reaction (~99% conjugation) was seen after 24 h of reaction ($p > 0.05$ after 24 h) (Fig. 2 (top)). Reaction rate of the CS PA to AGE increased with increasing AGE:CS ratio and 100:1 and 1000:1 AGE:CS ratios followed one-phase association kinetics (Table 2).

Conjugation of CS to acrylic acid was also monitored over time. No clear relationships were found between reaction time and conjugation for any acrylic acid:CS ratios ($R^2 < 0.3$ for one-phase association curve fits) (Fig. 2 (bottom)). Conjugation significantly increased after 6 h of reaction for all ratios ($p < 0.001$) but decreased again after 24 h of reaction ($p < 0.01$). Although conjugation increased again after 48 h, this increase was not significantly different from the 6 h conjugation ($p > 0.05$) ($n = 3$, 2-way ANOVA with Bonferroni Post-test). These results indicate that the CS–acrylic acid conjugation cannot be significantly increased with increased reaction time.

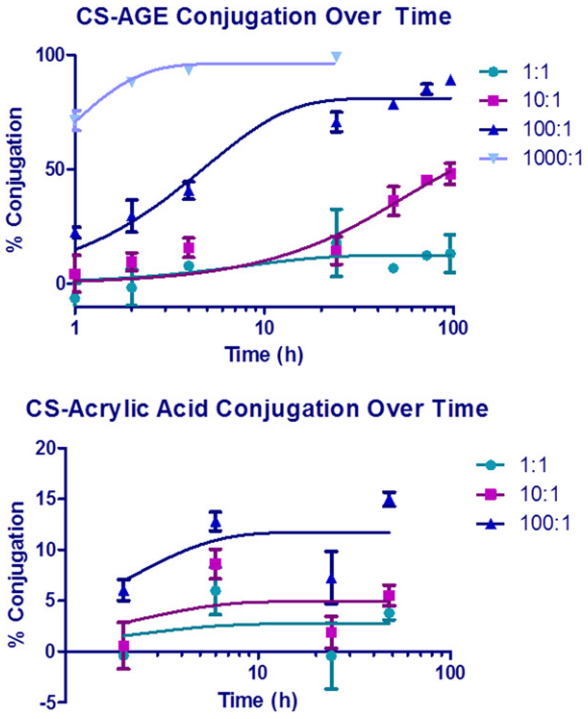


Fig. 2. (Top) CS–AGE conjugation over time for varying AGE:CS molar ratios with one-phase association exponential curve fit ($n = 3$). (Bottom) CS–acrylic acid conjugation over time for varying AA:CS molar ratios with one-phase association exponential curve fit ($n = 3$, $R^2 < 0.3$ for one-phase association curve fits).

3.2.2. *Qualitative ^{1}H NMR and ATR-FTIR study of vinyl incorporation in AGE–CS conjugates*

^{1}H NMR spectra for 1000:1 AGE–CS samples reacted at 0, 2, 4, 6, and 24 h were investigated for the incorporation of the AGE monomer on CS. Several classes of protons were resolved and assigned on the basis of their chemical shifts for CS and AGE (Fig. 3 (top)) and a good correspondence was seen between literature CS-4 spectra and that obtained in these studies (Toida, Kakinuma, Toyoda, & Imanari, 1994; Toida, Toyoda, & Imanari, 1993). Peaks corresponding to CS protons maintained their position (Fig. 3 (top)) as well as integrated area (Fig. 3 (bottom)) over the 24 h reaction period indicating stability of the overall CS structure throughout the reaction. In addition, no epoxide peaks in the 3.0–2.5 ppm region were evident, indicating thorough purification of un-reacted AGE monomer (Fig. 3 (top)). Inspection of the 7.0–5.0 ppm region of the AGE–CS spectra (Fig. 4 (top)) reveals the progressive incorporation of peaks corresponding to the vinyl AGE protons, AGE-1 and AGE-2.

In a qualitative analysis, a relationship was estimated between the AGE vinyl monomer and CS molecule in the sample of 1000:1 AGE:CS reaction. The peak at 2.0 ppm was assigned to the acetamide methyl protons of the GalNAc sugar (GalNAc-7). Integration of this peak was set to 100 and corresponds to three protons for the methyl group. For CS with MW 22,000 g/mol as assumed in this study, there are approximately 44 dimers (MW per dimer is 502 g/mol). Each dimmer has one methyl group arising from the GalNAc, therefore, the integrated area of peak GalNAc-7 is attributed to approximately 44 methyl groups, resulting in an integrated area of approximately 2.27 per methyl. As methyl has 3 protons, this corresponds to an integrated area of 0.75 corresponding to one proton. To estimate AGE vinyl group incorporation into CS, the integrated area of the AGE-2 proton, attributed to one proton, was ratioed to that of a single proton arising from CS (GalNAc-7, calculated at 0.75 integrated area for 1 proton) (Fig. 4 (bottom)). With increasing reaction time, vinyl:CS ratio increases from 0.51:1, 0.67:1, 0.77:1, 0.84:1 and 1.72:1 for 0, 2, 4, 6, and 24 h reaction times respectively (Fig. 4 (bottom)). A ratio above 1, as seen for the 24 h reaction, indicates the incorporation of more than one vinyl to each CS chain.

In order to further investigate the incorporation of AGE into CS and possible side reactions, ATR-FTIR spectra were obtained for AGE:CS 1000:1 molar ratio reactions over a 24 h reaction

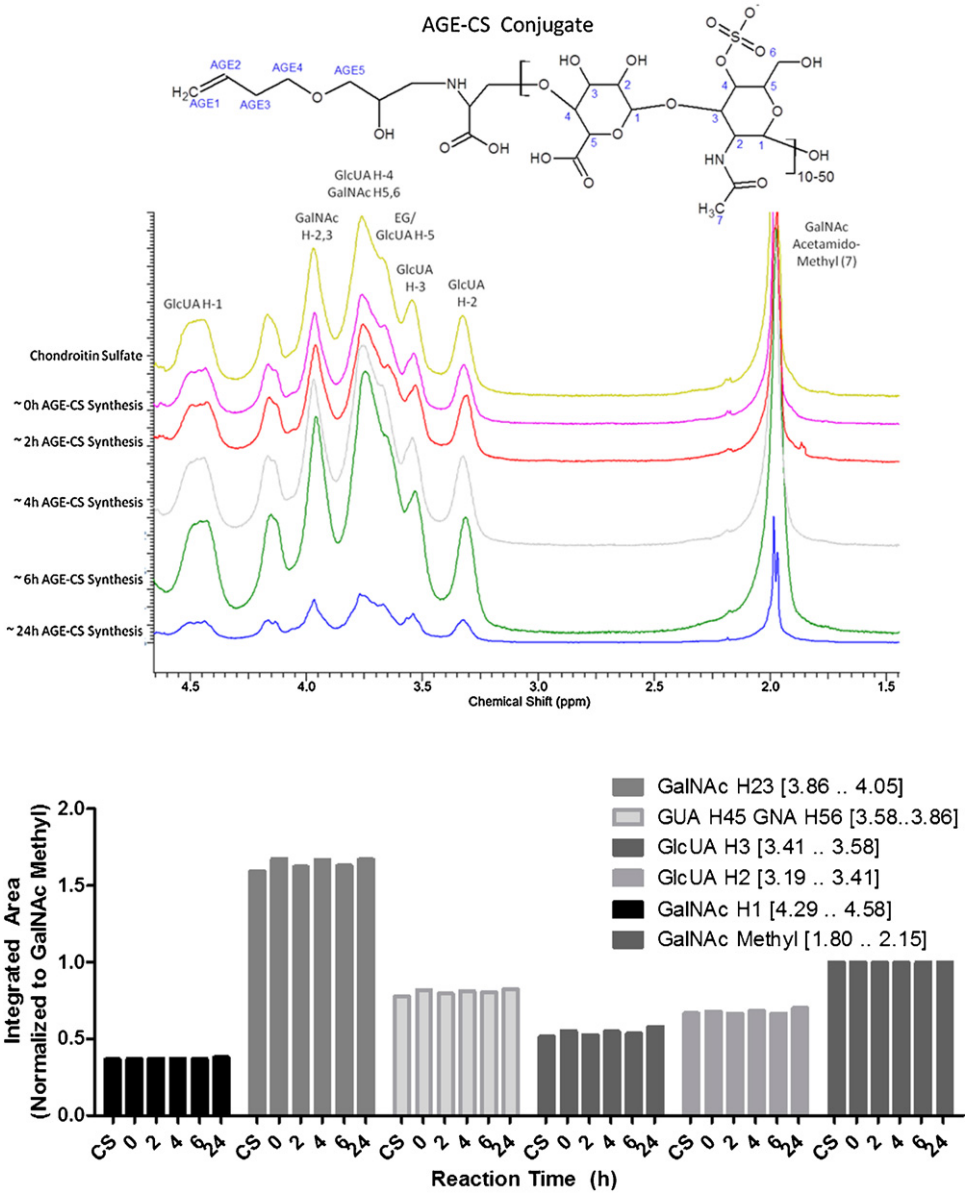


Fig. 3. (Top) CS region of ^{1}H NMR spectra of AGE–CS conjugate (1000:1 AGE:CS molar ratio) with increasing reaction time. (Bottom) Analysis of ^{1}H NMR integrated area (normalized to GalNAc methyl proton) of 1000:1 AGE:CS samples over 24 h reaction time.

period. Wavenumber assignments for key peaks corresponding to CS (Cabassi, Casu, & Perlin, 1978; Foot & Mulholland, 2005; Mathews, 1958; Orr, 1954) and AGE (Huang, Huang, Wang, Chen, & Chang, 2009) are presented in Fig. 5. Of particular interest is the C—O stretching mode in CS where it has been demonstrated that shifting occurs with changes to the hydroxyl group orientation (Orr, 1954). The most likely side reaction to occur would be with the primary hydroxyl of the CS GalNAc disaccharide (Parker & Isaacs, 1959), however this group is not clearly resolved in the ^{1}H NMR spectra (combined in the 3.58 and 3.86 ppm band), necessitating a secondary means of detection. ATR-FTIR spectra for AGE–CS at all reaction time points were consistent with that of natural CS (Fig. 5) (Cabassi et al., 1978; Foot & Mulholland, 2005; Mathews, 1958; Orr, 1954). No strong peaks corresponding to the AGE monomer were detected in the AGE–CS spectra (Fig. 5A and B). Spectral peak shifts may be expected in the 2850–3000 cm^{-1} CH_2=CH_2 stretching mode region with the addition of the AGE monomer to the CS chain, however, due to the relatively small size of AGE and the availability of only a few reactive sites, this contribution could not be discerned.

The FTIR peak centered at 1033 cm^{-1}, which arises from the C—O stretching mode in CS, was not significantly shifted in 2, 4, and 6 h reaction samples (ranging from 1031 to 1029 cm^{-1}), however, after 24 h of reaction, a larger shift was seen in this peak, with peak maxima shifting to 1024 cm^{-1} (Fig. 5A–D). Spectral shifts in this region would be expected to occur with changes to the hydroxyl group orientations in CS (Orr, 1954), and therefore, the shift in this spectral region for 24 h samples indicates possible CS hydroxyl group side chain reactions at this time point.

3.3. *Attachment of CS to amine-reactive substrates for the "grafting-to" synthesis strategy*

To further investigate CS binding to amine-reactive surfaces *via* the CS terminal primary amine functionality, epoxy-functionalized and non-functionalized (control) glass slides were treated with CS solutions of varying concentrations and CS attachment determined *via* contact angle measurements (Fig. 6). A decrease in contact

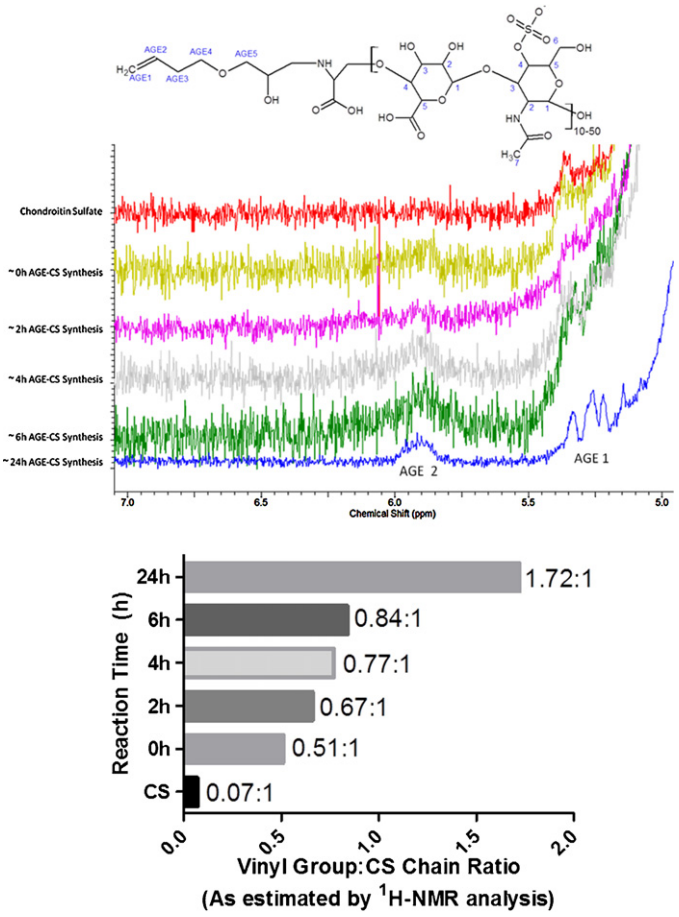


Fig. 4. (Top) ^{1}H NMR spectra of vinyl-proton region of AGE–CS (1000:1 molar ratio) samples synthesized over 24 h with AGE vinyl proton spectral assignments. (Bottom) Qualitative analysis of vinyl group incorporation into CS based on ^{1}H NMR spectral analysis of 1000:1 AGE:CS conjugation over time. Vinyl proton incorporation was based on the integrated area of proton 2 of AGE compared the integrated area of 1 proton, estimated from the methyl peak at 1.9 ppm.

angle is expected as CS is deposited onto the substrates since CS is a charged molecule and will attract water making the surface more hydrophilic. CS concentrations of 0, 0.125 and 2 mg/ml were investigated. Slides were rinsed thoroughly after treatment with CS to remove any non-covalently bound CS. On epoxy-functionalized slides, contact angle without CS was measured at $76.6 \pm 2.8^{\circ}$. With the addition of CS at 0.125 mg/ml contact angle was reduced to $63.4 \pm 9.5^{\circ}$ and further reduced to $38.2 \pm 6.6^{\circ}$ with the addition of CS at 2 mg/ml indicating an increase in hydrophilicity associated with the deposition of charged CS on the epoxy-functionalized surface (Fig. 6). Contact angle was significantly different on epoxy slides treated with CS with a significant decrease in contact angle seen as CS concentration increased ($p < 0.01$). Non-functionalized glass slides did not have a significant change in contact angle with CS treatment ($p > 0.05$).

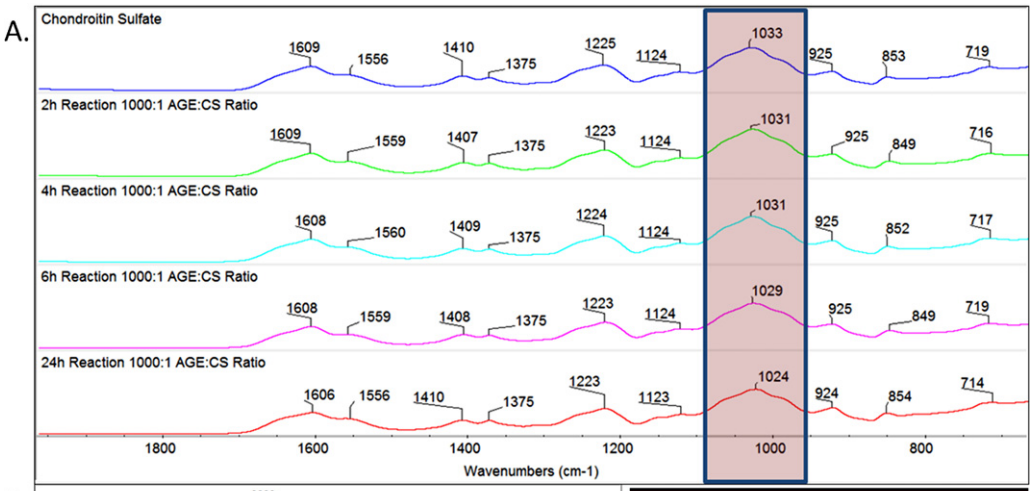
4. Discussion

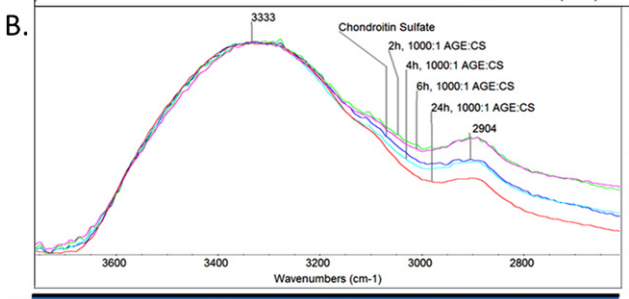
A major hurdle in the synthesis of polymers with carbohydrate pendant groups (glycopolymer) is the synthesis and preparation of the reactive carbohydrate or glycomonomer (Baskaran, Grande, Sun, Yayon, & Chaikof, 2002; Grande et al., 2000; Grande, Baskaran, & Chaikof, 2001; Sun, Grande, Baskaran, Hanson, & Chaikof, 2002). The glycomonomer must either be reactive to polymeric substrates or be polymeriziable into a polymeric backbone (Spain, Matthew, & Neil, 2007). Prior to the current study, high molecular weight, natural chondroitin sulfate had not been investigated as a glycol-monomer for glycopolymer bottle brush synthesis. In this report, we have identified a terminal primary amine in commercially available CS and determined the ability to covalently link CS to synthetic materials *via* this terminal-end functionality. Two amine-reactive chemistries were investigated for reaction with the terminal PA of CS and the attachment of polymerizable vinyl groups. The epoxide-amine chemistry was further investigated for the immobilization of CS onto amine-reactive surfaces.

4.1. CS terminal primary amine

CS is primarily obtained by isolation from tissue sources. It is inhomogeneous across tissue sources, as well as across isolation techniques and a minimal amount of data is provided on the physical and chemical properties of the biomolecule when purchased (Lauder, 2009; Volpi, 2007). For our studies, we required that a primary amine be available on the terminal end of CS. Although it is likely a serine residue is present based on reported cleavage techniques to remove CS from the protein backbone, exact isolation technique and protein residue content is not reported for commercially available CS (Anderson et al., 1965; Volpi, 2006, 2007). Variations in the isolation procedure may result in the introduction of additional PAs throughout the CS chains as well as cleavage of the linkage region from the CS main chain (Volpi, 2006). Although these small variations are not generally disruptive to the overall structure and function of the CS molecule and therefore not reported, they are critical for our investigation.

CS was investigated from two vendors, Calbiochem and Sigma, and three tissue sources, bovine trachea, bovine cartilage and shark cartilage. CS-6 from shark cartilage which is a larger type of CS (~65 K MW) had the highest number of primary amines per chain. Due to the larger size of this CS as well as its high PA content, it was not pursued for the synthesis of biomimetic aggrecan. However, with further purification, and mild isolation techniques, the utilization of CS-6 in biomimetic aggrecan may allow for a diverse family of biomimetic aggrecans to be synthesized with varying CS chemistry and molecular weight. The PA content of CS-4 and mixtures of CS from bovine trachea varied between vendors (Table 1). The difference in the number of PAs is indicative of the inhomogeneity of the CS isolation techniques. Calbiochem CS, which is a less pure mix of different CS isomers, may have more PAs due to protein impurities within the CS isolate. Also possible is the deacetylation of the GalNAc of CS. The amide bond in GalNAc is subject to hydrolysis in alkaline solutions, and processing conditions may cause breakage of this bond exposing PAs throughout the CS backbone. This is particularly disruptive to the synthesis of CS bottle brush structures since reaction chemistries must be limited to a single point on the molecule, preferably on the terminal end in order to develop a well controlled, organized structure. CS-4 from bovine cartilage, supplied by Sigma (C6737) was determined to have ~1.15 primary amines per CS chain. This is indicative of a single PA per CS chain, and based on isolation techniques, the single PA is likely at the terminal end of CS and arises from a serine residue. Sigma C6737 which is the sodium salt of chondroitin sulfate is marketed as a standard for cetylpyridinium chloride (CPC) titration and is therefore likely more purified than other marketed CS with reduced protein contamination. Mass Spectrometry techniques may also be used to investigate glycosaminoglycans and glycosaminoglycan oligosaccharides for their terminal end groups however these techniques generally require a high degree of material processing and can be hampered by the large size and charged nature of chondroitin sulfate (Postma et al., 2006; Venkataraman, Shriver, Raman, & Sasisekharan, 1999; Zaia, 2008). The method presented here is a simple and inexpensive means to determine the presence of terminal primary amines in CS samples.





Chondroitin Sulfate (CS)

Chemical Group	Wavenumber (cm^{-1})
S=O	1250-1240
C-O-S vibration	850-820; 928; 725
Hydroxyl (OH) Stretching Vibration	3200-3600
Amide 1 Band (carboxyl)	1630
C-C vibrations	1150 and 1050
C-H Vibrations	1420 and 1380
C-O Stretching Mode	1030
Monosubstituted Amide	1648, 1560, 1315
Associated Acetamido Methyl	1375

C.

Allyl Glycidyl Ether (AGE)

Chemical Group	Wavenumber (cm^{-1})
Epoxide	911
Ether Stretching	1050-1150
CH_2=CH_2 stretching mode	2850-3000
Alkyl (R-CH_3) stretching mode	1647

Fig. 5. ATR-FTIR spectra for AGE–CS conjugates reacted over 24 h with wavenumber assignments. Highlighted is the C—O stretching mode associated with the CS disaccharide backbone.

In the calculation of CS PA content, polydisperse molecular weight distributions may affect the calculation of the ratio of PAs to CS molecules. Based on reported literature values, a CS MW of 22,000 was assumed for CS-4 and mixed populations of CS (Muir, 1977; Volpi, 2007) and 65,000 for CS-6 however it is expected that there is a MW distribution within each CS sample and variations between vendor isolation techniques, tissue source and age may result in varying CS MW. If MW varies between products the calculation of the number of PAs per CS molecule may be skewed contributing to the discrepancy between calculated values. Based on the fluorescamine analysis of CS PA content conducted here, Sigma CS-4 from bovine cartilage, supplied by Sigma was chosen for further investigation.

4.2. Reactivity of the CS terminal primary amine and attachment of a polymerizable monomer

Two different amine reactive chemistries were investigated for the functionalization of CS at its terminal primary amine with a polymerizable monomer. Epoxide and carboxylic acid reactive groups were investigated by the conjugation of CS to allyl glycidyl ether and acrylic acid monomers respectively. Both monomers have one amine reactive functionality as well as a reactive vinyl group. The reactive vinyl group may be used subsequently in order to polymerize CS into brush structures *via* the "grafting-through" strategy (Ladmiral, Melia, & Haddleton, 2004; Okada, 2001; Spain et al., 2007). As the molar ratios of monomer:CS was increased, an increase in the percent of monomer conjugation was also seen (Fig. 2). Higher molar ratios could not be achieved due to insolubility of the monomers at higher concentrations. A large molar excess is likely required due to the small concentration of PAs available in comparison to the CS disaccharide concentration.

The carboxylic-acid amine reaction was investigated for its temporal dependence. Acrylic acid:CS conjugation only reached a maximum of 15% at a 100:1 molar ratio. This may be due in part to the several steps required to activate acrylic acid for reaction with PAs. An EDC/sulfo-NHS coupling reaction was utilized where incomplete activation of the carboxylic acids in acrylic acid may have led to fewer active monomers available for conjugation. Carbodiimide coupling of carboxylic acids and amines is often used

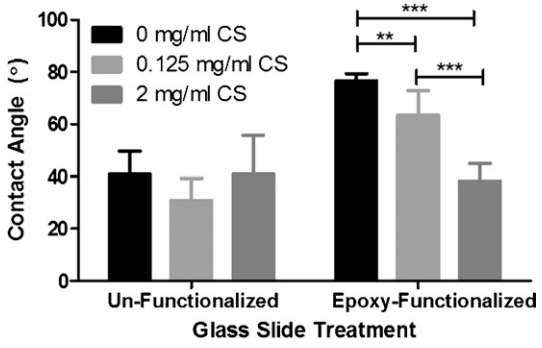


Fig. 6. Contact angle measurements on CS treated glass slides ($n > 4$, 2-way ANOVA with Bonferroni Post-tests, $^{**}p < 0.01$, $^{***}p < 0.001$).

in order to promote the reaction of the two functional groups in order to form amide bonds (Nakajima & Ikada, 1995). Amide bonds are very hydrolytically stable having a half life of *ca.* 600 years in neutral solutions at 25 °C which makes the amide linkage very attractive for bioconjugation applications (Kalia & Raines, 2010). In the absence of a coupling agent, carboxylic acids and amines do not form amide bonds however interactions between the two species can result in the formation of protonated amines. Protonated amines are not detectable using the fluorescamine assay. Their formation in the AA:CS reaction medium can result in a temporary decrease in the fluorescamine signal, transiently increasing the conjugation detected. This may account for the fluctuations in the conjugation seen over time. No clear time dependence was seen for the AA–CS reaction. Both linear and one phase exponential fits were poor (Fig. 2). No significant increase in conjugation was seen beyond 6 h of reaction. This is in good agreement with the window of reactivity of the EDC/sulfo-NHS activation of carboxylic acids which generally ranges from 2 to 4 h (Hermanson, 2008; Nakajima & Ikada, 1995). Modifications of the EDC/sulfo-NHS activation procedure may improve conjugation of the CS PA to carboxyl functional groups. A two-step activation procedure was utilized in this study in order to avoid activation of carboxylic acids in CS. Quenching of excess EDC with 2-mercaptoethanol may have reduced conjugation between CS and acrylic acid. Filtration of excess EDC instead of quenching may improve the reaction in addition to optimization of EDC/sulfo-NHS reagent concentrations. Due to the comparatively low conjugation between acrylic acid and CS seen in this study, the addition of a vinyl monomer to CS *via* acrylic acid conjugation is not optimal. These studies however do demonstrate the feasibility of coupling the CS PA with carboxylic acid functionalities using carbodiimide coupling.

The conjugation of AGE to CS was investigated and the reaction kinetics determined for varying AGE:CS molar ratios. At the molar ratio of 1:1 AGE:CS, no clear time dependence was observed (Fig. 2). Data could not be accurately fit to exponential or linear regressions ($R^2 = 0.4$ and 0.2 respectively). At low molar ratios, reaction between CS and AGE are minimal. This may be due to the large amount of CS present in the solution with respect to the terminal primary amine reactive group and the AGE monomer. For higher ratios, the CS–AGE reactions progressed with time and the rate constants increased with increasing AGE:CS molar ratio (Table 2). The 10:1 AGE:CS molar ratio reaction progressed with time but did not reach a plateau. Based on an exponential fit of moderate accuracy ($R^2 = 0.8$) this reaction is predicted to reach ~58.8% conjugation with a rate constant of 0.019 h^{-1} however the 95% confidence interval for the rate constant ranged from 0.002 h^{-1} to 0.036 h^{-1}. The extrapolated time constant had a 95% confidence interval between 28 h and 53 h and therefore may be beyond the scope of the time points investigated and thus the rate constant cannot be accurately determined from the time range investigated in this study. These studies were conducted at a CS concentration of 25 mg/ml; increasing the overall concentration of both CS and AGE monomer may aid in increasing reaction kinetics by reducing interpolymeric repulsion and increasing the coil–coil overlap time (O'Shaughnessy, 1994). Reaction kinetics at higher concentration however cannot be monitored using the fluorescamine assay due to the limited linear region of the fluorescamine signal to amine concentration standard curve.

At the 100:1 AGE:CS molar ratio, a maximum of 89% conjugation was reached after 96 h of reaction. Based on the one-phase association exponential fit ($R^2 = 0.93$), a plateau of 81% conjugation is predicted for this reaction with a rate constant of 0.2 h^{-1} corresponding to a time constant of 4.9 h (95% confidence interval between 4 h and 6 h). This indicates that a maximum conjugation was reached within the time window investigated. These studies at the 10:1 and 100:1 AGE:CS molar ratios demonstrate the longevity of the CS–AGE reaction. At the 1000:1 ratio, the reaction proceeds quickly at a rate constant of 1.3 h^{-1} reaching a conjugation of over 98% after just 4 h. In the synthesis of vinyl-ended CS chains, the highest conjugation is desired in order to synthesis a majority population of vinyl modified chains, therefore the 1000:1 ratio is most appropriate for this purpose. Studies at the lower molar ratios however demonstrate the ability to utilize the epoxide-amine reaction in synthesis techniques that require long reaction times such as the "grafting-to" method of brush synthesis (Advincula, 2004; Penn et al., 2002; Rühe et al., 2004).

We further investigated the incorporation of AGE monomer into CS (1000:1 AGE:CS molar ratio) over a 24 h reaction period using ^{1}H NMR (Figs. 3 and 4) and ATR-FTIR (Fig. 5). Both ^{1}H NMR and ATR-FTIR analysis of the AGE–CS macromolecule demonstrated stability of the CS chemical structure over the reaction period. Monitoring of vinyl group incorporation into CS suggested progressive incorporation of the AGE vinyl group onto CS with up to a 0.84:1 vinyl:CS ratio seen after 6 h. From this ratio it can be implied that approximately 84% of CS molecules have a single vinyl group incorporated at this reaction time point. In conjunction with fluorescamine data demonstrating reaction of AGE with the CS PA and ATR-FTIR data indicating minimal side reactions with CS hydroxyls; this data indicates, selective reaction of AGE with the CS terminal PA and incorporation of single vinyl groups at the terminal end of CS after 6 h of reaction. If a greater number of side reactions were occurring, the amount of vinyl proton incorporation into the CS ^{1}H NMR spectra would be expected to be considerably higher. An example of this can be seen in the 24 h reaction of AGE–CS (1000:1 AGE:CS molar ratio). After 24 h a ratio of 1.72:1 vinyl:CS was determined indicating, that after 24 h of reaction, more than one vinyl group is likely incorporated into each CS molecule. This would imply that side reactions of the AGE monomer with reactive groups other than the single terminal PA are occurring in these samples. This is further supported by the relatively large shift seen in the C—O stretching mode peak of the 24 h AGE–CS macromolecule, indicating possible side reactions on the CS hydroxyls. In the 1000:1 AGE:CS molar ratio reaction, the PA reaction rate was determined to be 0.75 h signifying that the majority of the primary amine reaction should occur within the first 3 h (~95% of the reaction). The plateau % conjugation for this reaction was high at 96% therefore, after the initial hours of reaction, very little PA is available and excess epoxide may be more likely to react with originally less reactive species. This phenomenon can be seen in the 24 h reaction.

The estimation of vinyl incorporation into the CS macromolecules is semi-quantitative and sensitive to CS MW, as well as noise in the ^{1}H NMR signal, signal base-lining and choice of integration region. However, in conjunction with fluorescamine studies and FTIR analysis, these results provide promising evidence that the AGE monomer is conjugating with CS specifically at the CS terminal primary amine. CS spectra after conjugation also matched well with natural CS spectra, indicating no major modification of the CS main chain with the AGE–CS monomer conjugation technique. No major peak shifts were detected at the 1033 cm^{-1} peak s of CS-macromolecules up to 6 h of reaction, indicating that there was no significant bonding of the CS hydroxyls by the AGE monomer at these time intervals and that incorporation of the AGE vinyl group into CS is limited to AGE reaction with the CS terminal PA (as detected by the fluorescamine assay).

In order to avoid over incorporation of vinyl groups throughout the CS backbone, the time of reaction must be monitored and limited. In this study, a 6 h reaction time of 1000:1 AGE:CS molar ratio reaction was demonstrated to have ~84% conversion of CS to vinyl-terminated CS. Further investigation of other AGE:CS molar ratio reactions (*i.e.* 100:1, AGE:CS) over the course of the reaction may reveal further possible time–concentration combinations that result in vinyl-terminated CS with minimal side reactions. The

simple modification of CS to incorporate terminal end polymerizable moieties was demonstrated here, and vinyl-terminated CS may be used in future studies in order to synthesize CS brush polymers *via* free-radical "grafting-through" polymerization strategies.

Vinyl–CS can be utilized in free-radical polymerization reactions for the fabrication of CS-glycopolymer structures *via* the "grafting-through" technique. Polymerization of glycopolymers and bottle brush polymers has been achieved through several mechanisms including ring-opening metathesis polymerization (ROMP) (Fraser & Grubbs, 1995), reversible addition–fragmentation chain transfer (RAFT) polymerization (Albertin, Stenzel, Barner-Kowollik, & Davis, 2006), atom transfer radical polymerization (ATRP) (Kohji, Yoshinobu, & Takeshi, 1998), cyanoxyl-mediated free-radical polymerization and classical free-radical synthesis (Baskaran et al., 2002; Grande et al., 2000, 2001; Sun et al., 2002) and may be amenable to the synthesis of CS bottle-brush structures.

4.3. Surface grafting of CS via the terminal primary amine

The utility of the CS terminal PA in immobilizing CS was investigated using model surfaces. Epoxy functionalized surfaces were treated with CS solutions of varying concentrations and investigated using contact angle measurements. AGE–CS conjugation studies suggested that epoxide reactions conducted at pH 9.4 up to 6 h were limited to reaction with the CS terminal primary amine. Therefore, attachment of CS to the epoxy-functionalized surfaces was likely mediated by epoxy–CS amine interactions.

Due to the charged nature of CS it is expected that surface hydrophilicity would increase (*i.e.* contact angle would decrease) as CS is deposited onto the surface. A decreasing contact angle was seen for epoxy-functionalized surfaces with increased CS concentration (Fig. 6). This indicates a strong relationship between CS concentration and attachment of CS to the surface suggesting covalent binding of CS. No such relationship was seen for non-functionalized glass surfaces, which is indicative of simply adsorbed CS. These surface studies provide evidence that CS can be immobilized onto amine reactive surfaces. Such immobilization can be transferred to synthetic polymer chains for the synthesis of CS bottle brush polymers *via* the "grafting-to" method.

5. Conclusions

The modification and subsequent immobilization of CS at its terminal end is critical for the fabrication of CS brush structures that mimic the bottle brush organization of proteoglycans such as aggrecan, where the bottle brush structure is critical for mechanical function. In this work, we have presented a simple method for the end-functionalization of natural, commercially available CS *via* a terminal PA. CS was prepared with a terminal vinyl functional group while maintaining the integrity of the CS biomolecule. Vinyl terminated CS can be used in future studies for the synthesis of biomimetic proteoglycan brush macromolecules *via* the "grafting-through" polymerization strategy. Epoxy-amine reactions were also utilized to immobilize CS to surfaces *via* its terminal PA demonstrating the utility of this reaction in the synthesis of brush structures *via* the "grafting-to" polymerization strategy. With the large diversity of proteoglycans and their implication in biological function, the techniques developed in this study can have far reaching effects in restorative and regenerative medicine.

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