Preparation and supramolecular recognition of multivalent peptide-polysaccharide conjugates by cucurbit[8]uril in hydrogel formation

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Abstract

Supramolecular hydrogels were fabricated by physically cross-linking phenylalanine functionalized polysaccharides with cucurbit[8]uril in water. We report a facile 2-step method of functionalisation of the polysaccharides hyaluronic acid (HA), carboxymethyl cellulose (CMC), hydroxyethyl cellulose (HEC) and guar with the dipeptide Phe-Cys. Addition of cucurbit[8]uril to the functional polysaccharides initiated physical cross-linking on account of strong 1:2 "hetero-ternary" complexes with the pendant Phe residues. In particular, HA and CMC based soft hydrogels displayed impressive viscoelastic behavior which was characterized using rheology, demonstrating accessibility to an array of material properties which would find broad applicability to many fields.

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Introduction

Polymer based hydrogels are becoming an increasingly important class of material and can be prepared by the introduction of polymer-polymer cross-links utilizing either covalent (chemical) or non-covalent (physical; i.e. metal-ligand complexation such as di- or tri- metal cations to polymer-bound carboxylates, analogous hydrogen bonding interactions such as those of the UPy motif, hydrophobic interactions as displayed in several thermogelling polymers such as PEG-PLGA copolymers, or host-guest interactions like those of cyclodextrin to adamantylamine or ferrocene) assembly approaches.[1–9] Increasing interest to fabricate hydrogels by physically cross-linking biopolymers such as polysaccharides has developed on account of their sustainable production, biocompatibility, biodegradable nature, responsiveness to enzymatic activity, process-ability, and commercial availability in a wide variety of molecular weights, from the reasonably low, 1.2 kDa, to the extremely high, 2.4 MDa in the case of hyaluronic acid for example.[10]

Polysaccharides are of particular interest as at low concentrations (i.e. 0.5-5 wt%) they express specific viscosity modifying characteristics in aqueous solution inherent to their structural chemistry, high degrees of polymer-polymer interactions and a structural variability not observed in polynucleotides and polypeptides. Therefore, they have already found many applications in food technology, cosmetic products and larger industrial processes such as fracking.[10] In terms of structural variance, many commodity polysaccharides are available that are chemically decorated with multiple glycol and carboxylate moieties, which significantly alter even dilute solution properties. Examples of their use in the formation of hydrogels through covalent cross-linking lower molecular weight polysaccharide species has shown good promise in recent reports, especially as 3D cell culture scaffolds and implantable drug delivery depots and can be considered strong competitors.[11,12] However, covalently cross-linked hydrogels, although strong, lack the ability to self heal once the network is broken through significant shear strain, and the material strength is dependent on the shortest chain, which experiences the most stress.[13–15] These shortcomings are being addressed by employing dynamic

and reversible non-covalent interactions as structural cross-links in hydrogels,[16] although introduction of dynamic binding moieties is rarely seen within polysaccharide chemistry, particularly with large molecular weight materials, as they are often difficult to functionalise on account of highly viscous reaction mixtures, large degrees of steric hindrance, and strong hydrogen bonding networks that render many reactive handles such as hydroxyl groups practically inert. Nevertheless, supramolecular hydrogels formed from polysaccharides are still desirable materials as they would have a high water content, exhibit rapid thixotropic behavior and other properties amenable to the development of shear-responsive and tunable soft matter devices.[17]

Our approach to fabricating such a material involves the host-guest chemistry of cucurbit[n]uril (n = 5–8 and 10; CB[n]). CB[n]s are a family of macrocyclic host molecules, which offer attractive supramolecular interactions for such applications. These hosts are methylene-linked oligomers of glycoluril that are symmetric 'barrel'-like molecules with two identical portal regions lined by ureido-carbonyl groups. The number (n) of glycoluril units determines the diameter of the CB[n] cavity without affecting the height of the molecular container (approximately 0.9 nm). These materials have demonstrated a low toxicity profile in a variety of models and are generally considered safe.[18–21] Smaller homologues of the CB[n] family (*i.e.* CB[5], CB[6] and CB[7]) are capable of binding single guests (typically cationic amines, metals, imidazolium ions and small molecule drugs).[22–25] In contrast to the smaller CB[n] homologues, CB[8] has a larger cavity volume (479 Å^3)[26,27] capable of simultaneously accommodating two planar and hydrophobic guests in a π-π-stacked geometry.[24,28–32] This host has been used most prominently in a 1:1:1 'hetero'-ternary complex using an electron-deficient first guest, such as methyl viologen (MV), and an electron-rich second guest such as naphthol, pyrene and dibenzylfuran.[30,32–34] In favorable cases, exceptionally high overall equilibrium binding affinities (K_{eq}(overall) = K_{eq}(1) $\times$ K_{eq}(2) up to 10^{14} M^{-2}) were reported,[26,30,33,35] leading to utilization in a number of applications ranging from the formation of diblock copolymers,[36–38] sequence-selective recognition of peptides,[39]

self-sorting systems,[40] surface modification,[41,42] protein conjugation,[43] to the formation of nanocapsules,[44] nanocomposites[45] and hydrogels.[46–49]

Urbach and coworkers first demonstrated that N-terminally charged aromatic amino acids, such as phenylalanine and tryptophan, bind in a 2:1 fashion forming a 'homo'-ternary complex with CB[8] through multiple non-covalent interactions acting synergistically. This results in exceptionally high equilibrium binding affinities (K_{eq} up to 10^{11} M^{-2}).[50] The ternary complex likely forms in a stepwise binding process whereby one amino acid guest enters first (K_{eq1}) followed by the second amino acid guest (K_{eq2}). These differ from the aforementioned hetero-ternary complexes as they do not yield a visible charge-transfer complex and the guest moieties do not carry a significant toxicity profile as they are naturally occurring amino acids. This 2:1 host-guest system has been utilized previously in a variety of systems including some of biological relevance such as the dimerization of proteins, which could be observed by FRET analysis,[51–54] in insulin sensing,[55] and also in our own previous study where these amino acids were used to physically cross-link cationic styrene polymers forming hydrogels with properties that could be finely tuned.[56]

In this study, our approach involves the chemical functionalisation of polysaccharides hyaluronic acid (HA), carboxymethyl cellulose (CMC), hydroxyethyl cellulose (HEC) and guar with a dipeptide, cysteine-phenylalanine (CF). This pendant dipeptide moiety exhibits strong, reversible, and stimuli-responsive CB[8]-based 2:1 'homo'-ternary binding motifs, which behave as cross-links between the polysaccharide chains, as depicted in Figure 1. Polysaccharide derived physical hydrogels are of great significance to the advancement of the materials science field as they are biocompatible, obtainable from renewable sources and of high molecular weight allowing for a very high water content. Indeed, many of these polysaccharides are already implicated in cell culture techniques, cosmetic products, and biomedical devices but their application to these areas is limited due to covalent crosslinking methods. Herein, we discuss the development of a mild and translatable method to function-alise high molecular weight polysaccharides with peptides, explore the contribution of the

different polysaccharide structures towards soft physically cross-linked materials, the rheological properties of the formed supramolecular hydrogels and the tunability of the materials by varying polysaccharide concentration.
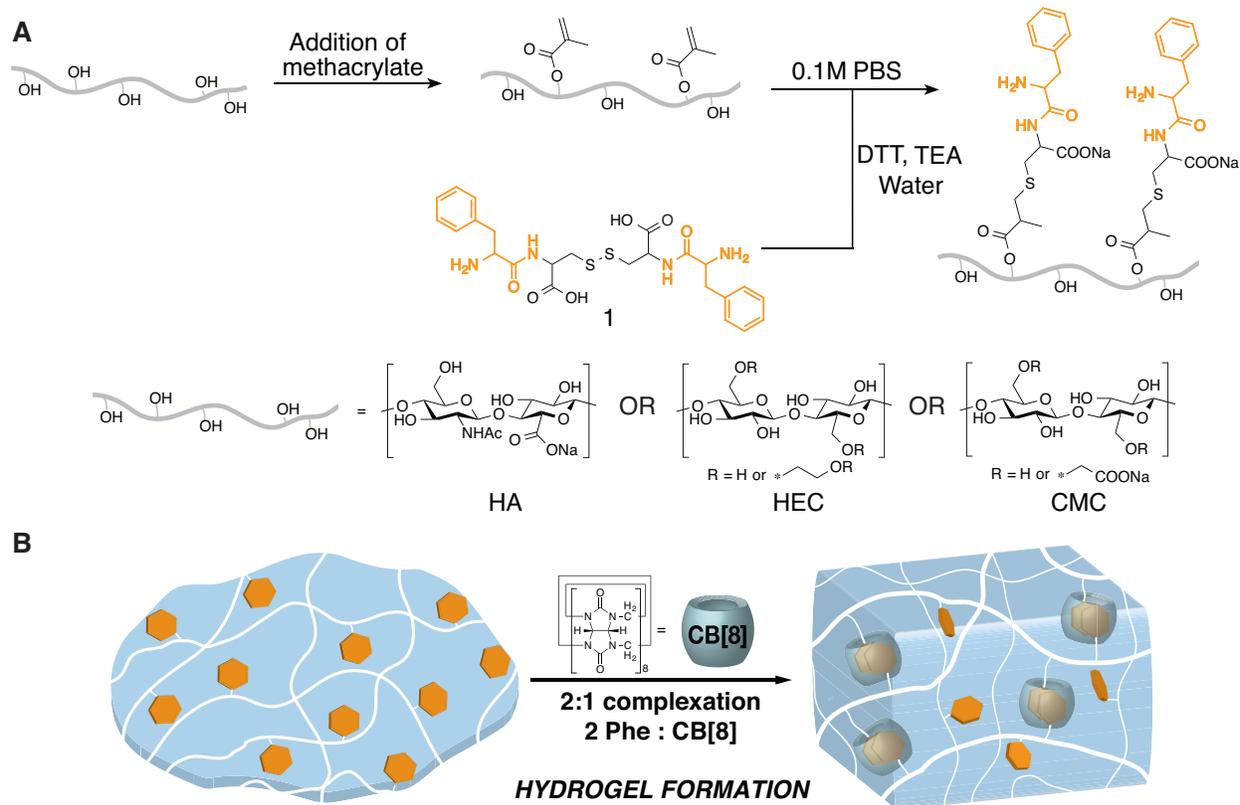

Figure 1: A: Methacrylate-bearing polysaccharides (HA-MA, HEC-MA and CMC-MA) were synthesized and the *in situ* reduction of the disulfide bridged CF peptide **1** in the presence of triethylamine facilitated Michael addition to yield polysaccharides with pendant CF CB[8] binding units. B: Schematic of hydrogel formation upon CB[8] addition. The phenylalanine residues of the pendant CF units (represented by the shaded hexagons) bind in a 2:1 fashion with CB[8]. The phenyl group of the terminal amino acid is encapsulated within the hydrophobic cavity by non-covalent interactions. Further interactions occur between the protonated *N*-terminus of the dipeptide unit and the CB[8] portal carbonyl groups. The CB[8] facilitated cross-linking results in a visco-elastic and shape persistent material.

Materials and methods

Chemicals were purchased from Sigma Aldrich. In all cases the highest molecular weight polysaccharides purchasable were used, specifically hyaluronic acid from Streptococcus zooepi-

demicus (1.5 MDa), carboxymethyl cellulose (700 kDa, MS 0.9, DS 0.9), hydroxyethyl cellulose (1.3 MDa, MS 2.5, DS 1.5) and guar (MW unspecified). ^{1}H-NMR (500 MHz) spectra were recorded using a Bruker Avance 500 Cryo Ultrashield. Chemical shifts were measured in ppm (δ) in D_2O with the internal reference set to δ 4.79 ppm. ^{13}C-NMR (125 MHz) spectra were recorded in D_2O. High resolution mass spectrometry was recorded using a Waters LCT ESI. Rheological characterization was performed using a TA Instruments DHR-2 controlled stress rheometer fitted with a peltier stage set to 20 °C. Dynamic oscillatory strain amplitude sweep measurements were conducted at a frequency of 10 rad s^{-1}. Dynamic oscillatory frequency sweep measurements were conducted at a 1% strain amplitude. All measurements were performed using a 40 mm parallel plate geometry with a gap of 0.500 mm and analyzed using TA Instruments TRIOS software. Cucurbit[8]uril was prepared according to literature procedures.[31] Dialysis of the polymers was carried out by placing the reaction solutions into a dialysis tube (Spectrum Labs, Spectra/Por, standard grade regenerated cellulose dialysis membrane 6, MWCO 15,000 Daltons) which was subsequently submerged in specified aqueous solutions. The external solutions were stirred at room temperature and replaced periodically over a 72 hour time period (ca. 4-5 times daily). The dialyzed polymer solution was then transferred into a round bottom flask, frozen with liquid nitrogen and lyophilized on a VirTis BenchTop Freeze Drier to yield fluffy solid materials. ITC measurements were carried out using a MicroCal Auto-ITC 200 (Malvern). The conditions applied were 25 °C temperature, 1000 rpm stirring speed, 60 s initial delay and 20 injections of 2 μl spaced by 90 s (the first injection was 0.4 μl) into the cell (270 μL). Peptide (1 mM) and CB[8] (50 μM) were dissolved in PBS at 25 °C. All the titrations were repeated three times and the blank (peptide in buffer) subtracted from previous analyses. Data were analyzed with MicroCal Origin software. The stoichiometry was checked with the "one binding site" model and then the data were fitted with the "sequential multiple binding site" model.

Synthetic procedures

Synthesis of 3,3'-disulfanediylbis(2-(2-amino-3-phenylpropanamido)propanoic acid) dihydrochloride (1, PheCys-S-S-CysPhe)

Boc-L-phenylalanine N-hydroxysuccinimide ester (Boc-Phe-OSu, 5.00 g, 13.8 mmol) was dissolved in DMF (50 ml) and triethylamine (5.75 ml, 41.4 mmol, 3 eq.) added. L-cystine dihydrochloride (1.72 g, 5.50 mmol, 0.4 eq), was added to the solution and stirred overnight. The solution was diluted with ethyl acetate and water and then acidified with 1.0 M $HCl_{(aq)}$. The aqueous layer was extracted and the organic layer was washed a further four times with acidified water. The organic layer was then collected and dried with magnesium sulfate, filtered and concentrated to dryness *in vacuo* to yield a yellow solid. The crude residue was dissolved in a minimal amount of chloroform and recrystallized overnight. The white crystals were collected by vacuum filtration, immediately suspended in dichloromethane (50 ml), and 4N hydrogen chloride in dioxane (50 ml) was added. The reaction mixture clarified on addition of the hydrogen chloride solution and the product precipitated within 2 hours. The reaction mixture was concentrated to dryness *in vacuo* and the white solid suspended in diethyl ether. The suspension was stirred until the precipitate appeared finely dispersed and then collected by vacuum filtration. Copious amounts of diethyl ether were washed over the solid, which was then dried under high vacuum and stored at 4°C. Yield: 2.510 g, 75 %; ^{1}H-NMR (D_2O, 500 MHz) δ (ppm) = 7.40-7.22 (6H, m, Ar-H), 7.22-7.15 (4H, m, Ar-H), 4.65-4.55 (2H, dd, J = 8.4 Hz, 5.1 Hz, C-H), 4.27-4.18 (2H, dd, J = 7.0 Hz, 7.0 Hz, C-H), 3.25-3.07 (6H, m, HC-H) 2.95-2.85 (2H, dd, J = 14.4 Hz, 8.4 Hz, HC-H). ^{13}C-NMR Spectroscopy (D_2O, 125 MHz) δ (ppm) = 173.12 (CO), 168.95 (COOH), 133.50 (ArC), 129.43 (ArCH), 129.15 (ArCH), 128.03 (ArCH), 54.26 (CH), 52.18 (CH), 38.40 (CH$_2$), 36.75 (CH$_2$), HRMS: calculated mass for $[C_24H_{31}1N_4O_6S_2]^+$: 535.1685, observed mass: 535.1697

General procedure for functionalization of polysaccharides with methacrylic anhydride (HA, HEC, guar)

Methacrylate addition procedure adapted from that published by Messager *et. al.* for low molecular weight HA.[57] Polysaccharide (1 g) was dissolved in 50 ml water. Dimethylformamide (DMF, 30 ml) was added and the solution stirred until homogeneous. The polymer solution was then cooled to 5 °C in an ice/water bath and methacrylic anhydride (740 μL, 5.00 mmol) added drop-wise over a period of fifteen minutes. The reaction mixture was removed from the ice bath and the pH maintained between 8-10 for 4 h by addition of 0.5 M NaOH(aq) before being left to stir overnight. The reaction mixture was then transferred directly to dialysis tubing (MWCO 15 kDa) and dialyzed against water for 5 days. The polysaccharide was then obtained by lyophilization in yields upwards of 70 % and functionalization with methacrylate observed by ^{1}H NMR.

Hyaluronic acid-methacrylate (HA-MA)

Yield: 880 mg, 88 %; Degree of MA functionalization: 15 %; ^{1}H NMR: (D$_2$O, 500 MHz) δ (ppm) = 6.21-5.97 (1H, s, vinyl-*H*), 5.75-5.58 (1H, s, vinyl-*H*), 4.56-2.57 (36H, br, polysaccharide backbone), 2.14-1.69 (9H, s, -NHCOC*H*$_3$ and 3H, s, allyl-*H*).

Hydroxyethyl cellulose-methacrylate (HEC-MA)

Yield: 871 mg, 87 %; Degree of MA functionalization: 10 %; ^{1}H NMR: (D$_2$O, 500 MHz) δ (ppm) = 6.16-6.06 (1H, s, vinyl-*H*), 5.74-5.63 (1H, s, vinyl-*H*), 4.63-2.97 (117H, br, polysaccharide backbone), 2.02-1.75 (3H, s, allyl-*H*).

Guar-methacrylate (Guar-MA)

Yield: 703 mg, 70 %; Degree of MA functionalization: 8 %; ^{1}H NMR: (D$_2$O, 500 MHz) δ (ppm) = 6.19-6.08 (1H, m, vinyl-*H*), 5.76-5.66 (1H, s, vinyl-*H*), 5.04-4.86 (8H, s, polysaccharide backbone) 4.37-3.32 (138H, br, polysaccharide backbone), 1.95-1.83 (3H, s, allyl-*H*).

Carboxymethyl cellulose-methacrylate (CMC-MA)

Procedure adapted from that published by Reeves *et. al.*[58] Carboxymethyl cellulose sodium salt (1 g) was dissolved in 50 mM sodium bicarbonate solution (200 ml) and stirred. 2-aminoethyl methacrylate hydrochloride (184 mg, 1.11 mmol) was added portion-wise to the polymer solution followed by N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 211 mg, 1.10 mmol). The reaction was stirred for 2 h and then further 2-aminoethyl methacrylate (184 mg) and EDC (211 mg) was added. The reaction was stirred for another 2 h and then precipitated in acetone. The polymer was collected by centrifugation, dissolved in water and dialyzed against water for 5 days before isolating by lyophilization. Yield: 716 mg, 72 %; Degree of MA functionalization: 12 %; ^{1}H NMR: (D_2O, 500 MHz) δ (ppm) = 6.17-5.89 (1H, s, vinyl-H), 5.77-5.56 (1H, s, vinyl-H), 4.58-2.91 (71H, br, polysaccharide backbone), 2.00-1.74 (3H, s, allyl-H).

General procedure for Michael addition of Phe-Cys to PS-MAs

All polysaccharide-cysphe (PS-CF) materials were produced *via* the following method. Polysaccharide-methacrylate (PS-MA, 400 mg) was dissolved in PBS solution (100 mM, 100 ml) and degassed with nitrogen for 1 h in darkness. Separately, **1** (929 mg, 1.54 mmol, ∼10 molar equivalents to MA residues) was dissolved in water (5 ml) and triethylamine (1.28 ml, 9.24 mmol, 6.00 eq) added. The peptide precipitated on addition of the base and then re-dissolved. The solution was degassed with nitrogen for 15 min before the addition of DL-dithiothreitol (DTT, 235 mg, 1.53 mmol, 1 eq). The reaction mixture was stirred for 4 h and then injected into the degassed PS-MA solution and stirred continuously overnight. The solution was then transferred to dialysis tubing and dialyzed against brine for 3 days and then water for 2 days. The functional polysaccharide was then isolated by lyophilization, degree of functionalization estimated by ^{1}H NMR and the consumption of methacrylate observed to be 100 %.

9

Hyaluronic acid-cysphe (HA-CF)

Yield: 332 mg, 83 %; Degree of CF functionalization: 10 %; ^{1}H NMR: (D$_2$O, 500 MHz) δ (ppm) = 7.47-7.17 (5H, m, aryl-H), 4.57-2.53 (65H, br, polysaccharide backbone), 2.16-1.73 (15H, s, -NHCOCH_3), 1.24-1.04 (3H, s, CH_3).

Hydroxyethylcellulose-cysphe (HEC-CF)

Yield: 392 mg, 98%; Degree of CF functionalization: 9 %; ^{1}H NMR: (D$_2$O, 500 MHz) δ (ppm) = 7.51-7.19 (5H, m, aryl-H), 4.57-2.33 (113H, br, polysaccharide backbone), 1.27-1.06 (3H, s, CH_3).

Guar-cysphe (Guar-CF)

Yield: 364 mg, 91%; Degree of CF functionalization: 10 %; ^{1}H NMR: (D$_2$O, 500 MHz) δ (ppm) = 7.43-7.19 (5H, m, aryl-H), 5.02-4.88 (7H, s, polysaccharide backbone), 4.37-2.60 (97H, br, polysaccharide backbone), 1.27-1.11 (3H, s, CH_3).

Carboxymethylcellulose-cysphe (CMC-CF)

Yield: 298 mg, 75%; Degree of CF functionalization: 9 %; ^{1}H NMR: (D$_2$O, 500 MHz) δ (ppm) = 7.47-7.08 (5H, m, aryl-H), 4.63-2.40 (97H, br, polysaccharide backbone), 1.26-1.04 (3H, s, CH_3).

Synthesis of N-(L-phenylalanyl)-S-methylcysteine (S-methyl-CysPhe)

Boc-L-phenylalanine N-hydroxysuccinimide ester (Boc-Phe-OSu, 2.10 g, 5.8 mmol) was dissolved in DMF (20 ml) and triethylamine (2.20 ml, 15.8 mmol, 2.7 eq.) added. S-methyl-L-cysteine (711 mg, 5.27 mmol, 0.9 eq), was added to the solution and stirred overnight. The solution was diluted with ethyl acetate and water and then acidified with 1.0 M HCl$_{(aq)}$. The aqueous layer was extracted and the organic layer was washed a further four times

with acidified water. The organic layer was then collected and dried with magnesium sulfate, filtered and concentrated to dryness *in vacuo* to yield an off-white solid. The crude residue was immediately suspended in dichloromethane (20 ml), and 4N hydrogen chloride in dioxane (20 ml) was added. After 4 h the reaction mixture was concentrated to dryness *in vacuo* affording an oily residue which was swirled in diethyl ether until a fine off white solid was formed. This was collected by vacuum filtration. Copious amounts of diethyl ether were washed over the solid, which was then dried under high vacuum and stored as the hydrochloride salt at 4°C. Yield: 1.300 g, 78 %; ^{1}H-NMR (D$_2$O, 500 MHz) δ (ppm) = 7.35-7.17 (5H, m, Ar-H), 4.52-4.46 (1H, dd, J = 8.2 Hz, 5.3 Hz, cysC-H), 4.42-4.19 (1H, dd, J = 7.1 Hz, 7.1 Hz, pheC-H), 3.20-3.14 (1H, dd, J = 14.1 Hz, 7.1 Hz, pheHC-H), 3.14-3.07 (1H, dd, J = 14.1 Hz, 7.1 Hz, pheHC-H), 2.96-2.87 (1H, dd, J = 14.0 Hz, 5.3 Hz, cysHC-H), 2.81-2.74 (1H, dd, J = 14.0 Hz, 8.2 Hz, cysHC-H), 2.04 (3H, s, -SCH_3. ^{13}C-NMR Spectroscopy (D$_2$O, 125 MHz) δ (ppm) = 173.30 (cys$C$O), 168.95 (phe$C$O), 133.53 (ArC), 129.43 (ArCH), 129.13 (ArCH), 128.00 (ArCH), 54.25 (phe$C$H), 52.23 (cys$C$H), 36.74 (phe$C$H$_2$), 34.58 (cys$C$H$_2$), 14.64 (-SCH$_3$). HRMS: calculated mass for [C$_1$3H$_1$9N$_2$O$_3$S]$^+$: 283.1115, observed mass: 283.1116

Hydrogel formation

Example procedure for 2 wt% hydrogel with 0.5 wt% CB[8]; 20 mg of polysaccharide-cysphe (PS-CF) was dissolved in 1 ml of PBS (0.01M) by stirring for several hours until a viscous transparent solution formed. CB[8] (5 mg) was added and the gels stirred (approximately 1 h) until homogeneously turbid and displaying viscoelastic properties.

Results and discussion

Functionalization of polysaccharides

Inspiration for our functionalization route of the high molecular weight polysaccharides came from that used to produce the commercially available 2D/3D cell culture material Glycosil®.[11] In this example, thiol-modified hyaluronic acid is cross-linked *via* Michael addition to polyethylene glycol diacrylate (PEGDA), creating covalent linkages between polymers. In the production of Glycosil®, acid-degraded hyaluronic acid (HA) is functionalized by coupling of 3,3'-dithiobis(propanoic hydrazide) to the carboxylic acids along the polysaccharide backbone. The thiol could then be exposed for Michael addition to the PEGDA by addition of common disulfide reducing agents such as dithiothreitol.[59] However, this route to functionalization is not immediately applicable to many other commodity polysaccharides such as hydroxyethyl cellulose (HEC) as they do not have pendant carboxylate units. This methodology is also not useful for very high molecular weight polysaccharides that have not undergone an acid-degradation step as the high viscosity of the reaction mixture severely inhibits the amide coupling reaction.

One common structural feature amongst all polysaccharides is a primary alcohol present from the C6 position. We therefore chose to pursue a functionalization route whereby the C6 primary alcohol would be used as a common handle of attachment to each polysaccharide. For HA, HEC and guar, conversion of the C6 primary hydroxyl groups to methacrylate esters was achieved by addition of methacrylic anhydride to the polysaccharide solution in a slightly basic environment. This method has been reported previously but only on low molecular weight or acid-degraded hyaluronic acid. We found that even under our highly viscous reaction conditions resulting from the high molecular weight HA, HEC (1.5 MDa and 1.3 MDa, respectively) and guar concentration, the reaction was still possible. However, careful control of the reaction pH around 8 was required to prevent the cleavage of the newly formed ester bonds. Raising the pH beyond 8 yielded PS-MAs with very low degrees of

substitution. Once optimized, degrees of functionalization of 10 % were readily achieved on all three polysaccharides (per monosaccharide unit) and the methacrylate functionality allows attachment of the smaller phenylalanine-derived thiol-bearing molecules, in our case the dipeptide Cys-Phe (CF), *via* Michael addition. In the case of guar, functional products were obtained but these were sparingly soluble and precipitated on addition of CB[8], giving evidence of supramolecular network formation but not strong enough to withstand synersis effects. Nevertheless, good degrees of functionalization were reproducibly possible across all polysaccharides as observed by ^{1}H NMR, Figure 2 (for ^{1}H NMR of guar, guar-MA and guar-CF, see Figure S1).

CMC is highly substituted on the primary alcohol with carboxymethyl units (supplier provided DS: 0.9) and therefore CMC was functionalized with methacrylate *via* a different method. Harnessing coupling reagent EDC and 2-aminoethyl methacrylate to create methacrylate functionality to the polysaccharide chain through the more prevalent carboxymethyl units[58] present on 90 % of the C6 oxygens. However, with all polysaccharides we found that the mild reaction conditions for Michael addition of Cys-Phe were appropriate for all polysaccharides and scalable to the order of grams, producing material in quantities far beyond the required analytical levels, Figure 1A. Degrees of functionalisation per monosaccharide were calculated by ^{1}H-NMR by comparing the aromatic signal to the broad signal associated with the protons of the polysaccharide backbone, and 100% methacrylate conversion was achieved in all samples without requiring further optimization between polysaccharides, Figure 2. Also noteworthy is the lack of resolution of the polysaccharide backbone proton signals, inferring minimal or no degradation of the polysaccharides throughout the functionalisation process. FTIR also proved to be a useful method of observing functionalisation, as in all PS-MA and PS-CF samples peaks between 1850 and 1700 cm^{-}1 were observed relating to the presence of ester bonds, Figures S5, S6, S7 and S8.
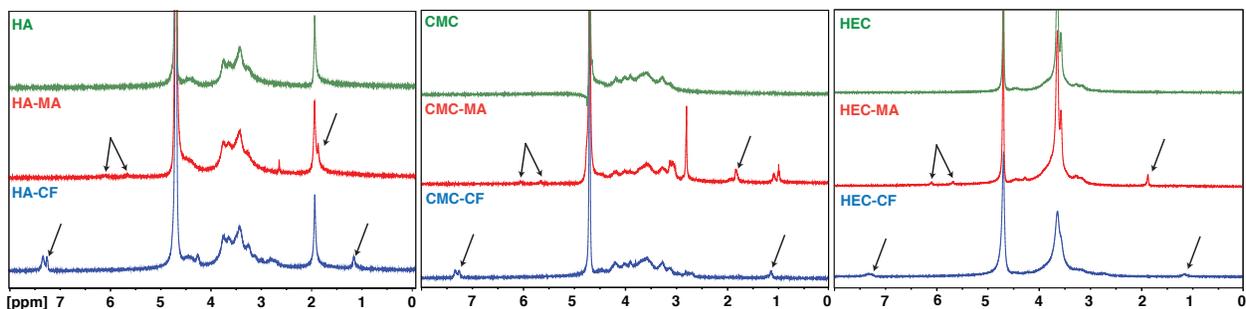
Figure 2: ^{1}H-NMR stacks of HA, HEC and CMC and their respective PS-MA and PS-CF derivatives. In all examples, functionalisation with methacrylate was achieved and Michael addition of CF dipeptide proceeded to 100% completion as proven by complete lack of the MA signals at 5.5-6.0 ppm in the PS-CF spectra.

Formation of Hydrogels

Facile preparation of the supramolecular hydrogels was achieved by the simple addition of solid CB[8] to phosphate buffered solutions of PS-CF. Preceding CB[8] addition, the PS-CF solutions were transparent, colorless and exhibited viscous flow when inverted. Upon addition of CB[8] the materials became turbid, and after a short period of stirring, expressed more elastic behavior as the materials gained rigidity and persistent shape, Figure 3. This is a consequence of the phenylalanine derivatives pendant from the polysaccharide chains forming 2:1 complexes with CB[8], physically cross-linking the polysaccharides, Figure 1B. The HEC based hydrogels experienced syneresis whereby the network shrunk over the period of just a few hours releasing water from the material, and were not stable enough to consider rheological analysis, Figure S2. However, this syneresis can be overcome by introduction of a small amount of either the HA-CF or CMC-CF to support the HEC network and this is currently being investigated within our laboratory along with further blending of other hydrogel formulations. Visually, the HA hydrogels appeared much stronger than the CMC materials, especially at 1 wt% polymer loading and the rheological properties of these two materials were further investigated.

The binding constants of the CF binding unit was also investigated using isothermal titration calorimetry (ITC), Figure 3D. To elucidate the thermodynamic parameters of binding,
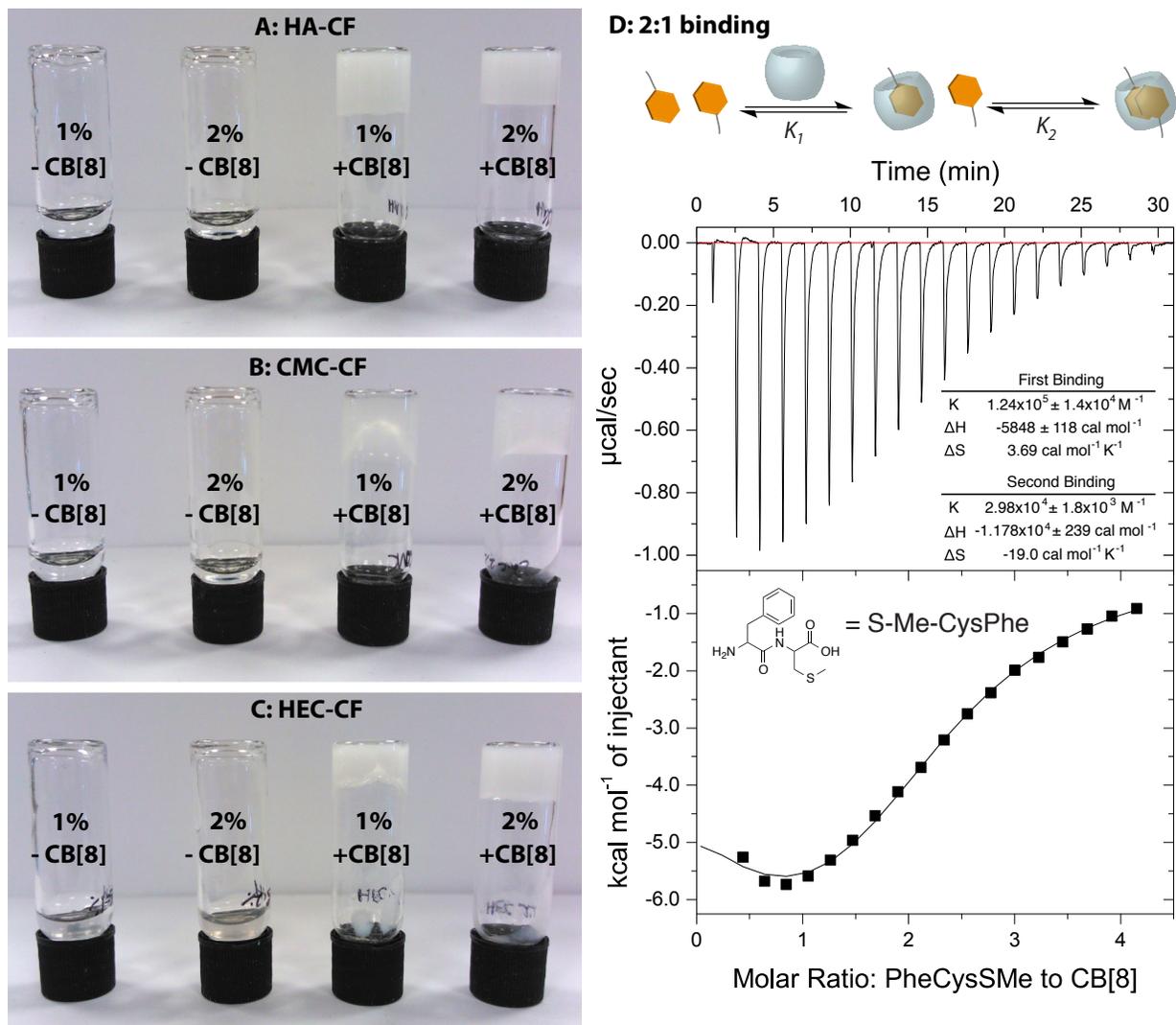
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A: HA-CF

1% - CB[8] | 2% - CB[8] | 1% +CB[8] | 2% +CB[8]

B: CMC-CF

1% - CB[8] | 2% - CB[8] | 1% +CB[8] | 2% +CB[8]

C: HEC-CF

1% - CB[8] | 2% - CB[8] | 1% +CB[8] | 2% +CB[8]

D: 2:1 binding

Time (min)

First Binding

K	$1.24 \times 10^5 \pm 1.4 \times 10^4$ M^{-1}
ΔH	-5848 ± 118 cal mol^{-1}
ΔS	3.69 cal mol^{-1} K^{-1}

Second Binding

K	$2.98 \times 10^4 \pm 1.8 \times 10^3$ M^{-1}
ΔH	$-1.178 \times 10^4 \pm 239$ cal mol^{-1}
ΔS	-19.0 cal mol^{-1} K^{-1}

= S-Me-CysPhe

Molar Ratio: PheCysSMe to CB[8]

Figure 3: A-C: Turbid hydrogels were formed at 1 and 2 wt% polymer loading in PBS (10 mM) on addition of 0.5 wt% CB[8]. Polysaccharide solutions without CB[8] were viscous but unable to form hydrogels. D: Binding stoichiometry and thermodynamic parameters determined by ITC employing a sequential binding sites model. The binding stoichiometry was determined to be 2:1 and overall binding constant to be 3.70 x 10^9 M^{-2}. 1 mM S-Methyl-CysPhe was injected into 50 μM at 25°C in 10 mM PBS.

a small molecule (S-methyl-CysPhe) was synthesized as a "model molecule". Methylation of the cysteine residue was required to prevent disulfide bond formation. An overall binding constant of 3.70 x 10^9 M^{-2} was determined using a multiple sequential binding sites model and a binding stoichiometry of 2:1 (CF:CB[8]) was observed. This binding constant is 2 magnitudes lower than the tripeptide PheGlyGly 2:1 binding to CB[8] previously reported by Urbach *et al.*[50] This can likely be attributed to the reduced distance and therefore higher electronic repulsion between the carbonyl-lined CB[8] portals and the C-terminus of the S-Methyl-CysPhe dipeptide. Nevertheless, this 2:1 binding was still strong enough to initiate supramolecular gelation.

Rheological Characterization

Oscillatory rheology

The amplitude dependent oscillatory rheology of the hydrogels was first investigated to estimate material strength and yield (gel-sol transition) points. Both HA and CMC hydrogels displayed broad viscoelastic regimes and control over the moduli was achieved through modulating polymer content (although this is also possible by moderating CB[8] concentration as demonstrated in a previous publication[56]), Figure 4A. Remarkably, both HA and CMC systems displayed identical G" in the range of 0.1-100 % oscillation strain at 1 and 2 wt% loading. However, the behavior of G' is starkly different between systems. In the case of 2 wt% HA-CF for example, G' (405 ± 5 Pa) is roughly double that of G" (175 ± 1 Pa) before the critical strain point (90 % strain) indicating highly elastic behavior with tan δ of 0.43 (tan δ = 0.62 for 1 wt%). The CMC hydrogels behave rather differently with the 1 wt% gels exhibiting G" > G' (tan δ > 1) and in the case of 2 wt% gels G' (221 ± 1 Pa) is only very slightly dominant over G" (184 ± 1 Pa), tan δ of 0.83 exhibiting a critical strain closer to 70%. In all cases the relatively high critical strains demonstrate that the materials have a high tolerance of strain deformation, likely accountable to persistent remodeling of the strong supramolecular network by continuous reorganization and equilibration of the
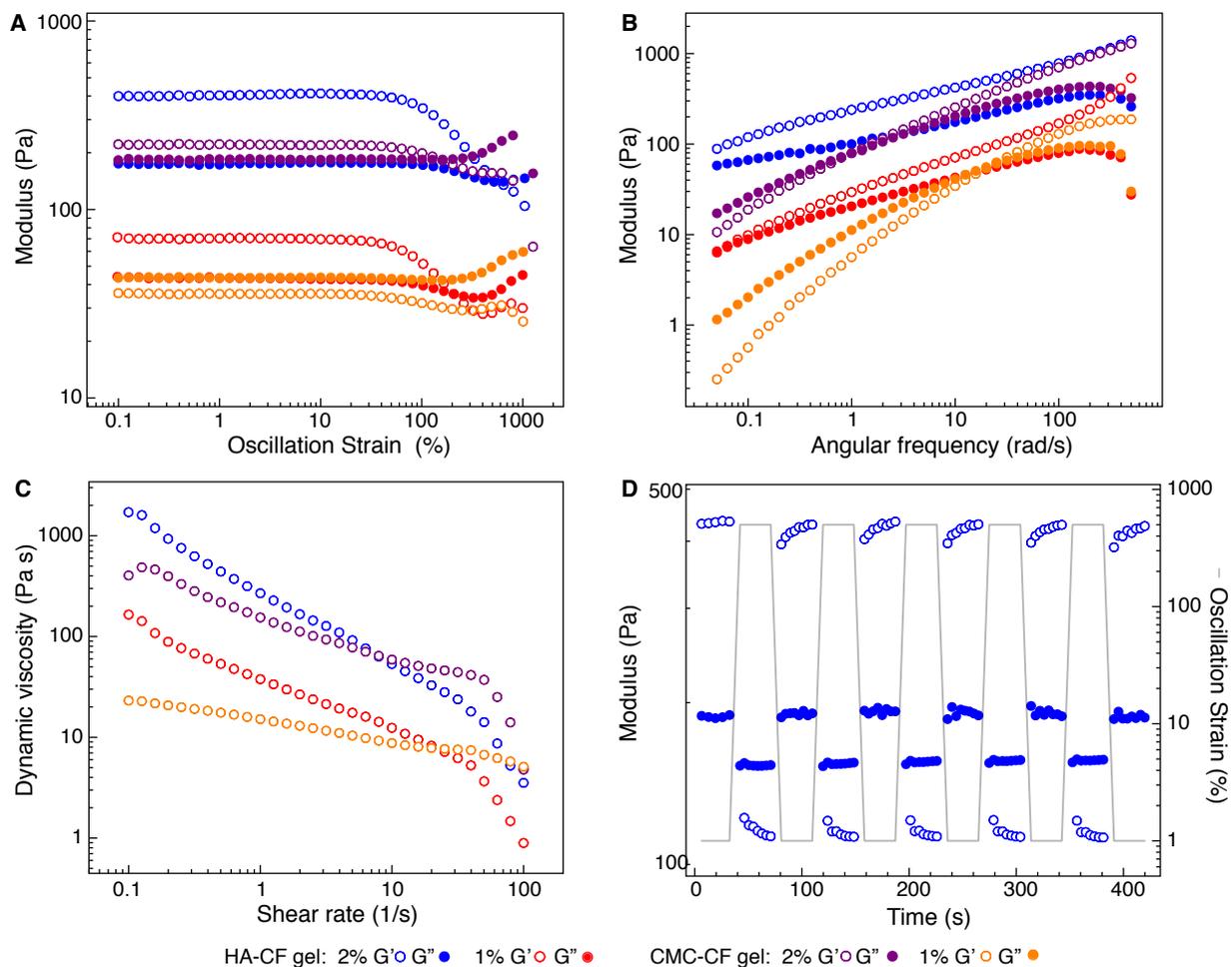
Figure 4: Oscillatory and shear dependent rheology of HA and CMC based hydrogels at 1 and 2 wt% polysaccharide loadings with 0.5 wt% CB[8]. A: Amplitude dependent oscillatory rheology. All materials expressed broad viscoelastic regimes and resisted yielding up to 90 % strain. B: Frequency dependent oscillatory rheology, demonstrating HA gels have stronger and more ordered networks than the CMC gels. C: Shear dependent rheology, increase in shear rate on the materials caused a drop in their viscosity on account of the mechanically induced dissociation of the CB[8] ternary complexes. D: Alternating strain experiment on HA-CF 2 wt% with CB[8] 0.5 wt%, applied oscillatory strain alternated between 1 and 500 % for 30 s periods. At high strain, G" dominates. Upon alternating back to 1 % strain, G' recovers rapidly and the original viscoelastic property is recovered. This process was repeated across five high strain periods demonstrating good recyclability.

17

CB[8] cross-links that have fast association kinetics. It is also notable that at high strains (200-1000%) the moduli appear to increase again but with G" > G'. This is likely attributed to the structural relaxation time whereby the rate of strain deformation is much higher than the rate of relaxation of the material, and therefore a substantial increase in the loss modulus is observed. On account of the high strain likely breaking all CB[8] mediated cross-links, the experienced stress maximum is likely to be in a similar time period as the highest velocity reached during the deformation process, i.e. at high strain the material is expressing a more viscous liquid-like behavior.

It is apparent from the frequency sweeps that the CMC hydrogels express a much weaker internal network at lower frequencies than the HA hydrogels, exemplified by G" > G' and relatively steep gradients, Figure 4B. A higher polymer loading moves the stress relaxation point (crossover point) to lower frequencies, as expected by more polymer-polymer interactions. However, the gradients expressed of CMC-CF materials are still reasonably steep and highly frequency dependent as the moduli cover two orders of magnitude compared to the HA-CF hydrogels. These differences in gradients demonstrate that HA-based gels are more structured and polymer-polymer interactions are more definite, i.e. the system is more static and so is less dependent on applied frequency and is approaching the behavior expected of a chemically cross-linked material. In terms of the CMC based hydrogel, the gradient is much steeper demonstrating a greater degree of reordering of the system at low frequencies, in this case the internal structure of the material is relatively weaker and more dependent on the CB[8] cross-links, polymer-polymer interactions are either not as common or not as strong. G' also dominates throughout the frequency sweep for the HA materials again inferring that the HA hydrogels are vastly more structured materials with less reordering on the molecular level. The difference in properties between the two is possibly accountable for by the larger molecular weight of the HA over CMC (1.5 MDa compared to 700 kDa) and therefore a higher propensity for such interactions. Interestingly, at higher angular frequencies the materials express similar behavior as the CB[8] cross-links begin to dominate the

18

viscoelastic response of the material regardless of polysaccharide choice, inferring that the material strength is highly dependent on the cross-link lifetime, which should be identical in these cases as the binding motifs are identical, and is in support of our previously published reports.[48,56]

Shear-dependent rheology

On account of the materials being assembled *via* many non-covalent interactions we expect these hydrogels to express shear-thinning behavior. Studying the dynamic viscosity as a function of shear rate on the materials, it is demonstrated that the viscosity of the material decreases as applied shear rate increases on account of the CB[8] facilitated cross-links dissociating as the material is deformed, Figure 4C. This type of behavior is important for injectable delivery systems where shear rates are typically very high as materials are extruded through syringe needles. Despite this, a rapid recovery rate of the material is equally important to minimize cargo loss on injection. Therefore, alternating strain experiments were also performed on all materials to examine the rate of thixotropy, Figure 4D and Figures S3 and S4. Strains of 1 and 500 % were applied to the materials for alternating 30 s periods. At 1 % strain all materials (2 wt% HA-CF and CMC-CF, 1 wt% HA-CF, all with 0.5 wt% CB[8]) expressed dominant G' as predicted by the earlier amplitude sweeps. At 500 % strain both G' and G" decreased but G' also moved below G", representing network breakdown and material "flow" as the CB[8] cross-links have sheared under the high strain condition yielding to a material that behaves as a viscous polymer solution. Alternating back to 1 % strain, the materials recover rapidly over the 30 s time period to their original G' and G" values owing to the fast kinetics of association of the phenylalanine units to CB[8]. This process was was repeated over 5 cycles with no loss in material property showing good recyclability. This demonstrates that the polysaccharide components do not get destroyed on a molecular level, there is no breakage of covalent bonds, during the application of 500 % strain accountable to the shearing of the CB[8] cross-links offsetting such a process.

19

Conclusion

We report a facile two-step method toward functionalising a variety of polysaccharides with short peptides. Our methodology is highly reproducible, translatable to a variety of polysaccharides, and causes no degradation of the polysaccharide high molecular weight backbones, a remarkable feat given the first step of most polysaccharides functionalisation is an acid-degradation to lower viscosity and improve reactivity. This has allowed for physical hydrogel formation on the addition of CB[8] at polymer concentrations of 1-2 wt%, much lower than many other current standards, which rely on lower molecular weight polysaccharides. Although only HA-CF and CMC-CF gave stable hydrogels on addition of CB[8], their rheology has been extensively explored and it is apparent these systems can be engineered to obtain materials with properties that can be pre-selected and then simply tuned in. This work has exciting prospects in the application of drug delivery and tissue engineering where shear-thinning, rapid thixotropic behavior and tunable moduli are paramount for success.

Acknowledgement

Supporting Information Available

Further supporting information and data can be found on-line. This includes NMR characterization of guar derivatives, IR characterization and further rheological analysis of hydrogel formulations.

This material is available free of charge via the Internet at `http://pubs.acs.org/`.

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