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Rational Design of Network Properties in Guest-Host Assembled and Shear-Thinning Hyaluronic Acid Hydrogels

Christopher B. Rodell, Adam Kaminski, and Jason A. Burdick*

Department of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania, 19104, United States

Abstract

Shear-thinning hydrogels afford direct injection or catheter delivery to tissues without potential premature gel formation and delivery failure or the use of triggers such as chemical initiators or heat. However, many shear-thinning hydrogels require long reassembly times or exhibit rapid erosion. We developed a shear-thinning hyaluronic acid (HA) hydrogel based on the guest-host interactions of adamantane modified HA (guest macromer, Ad-HA) and β -cyclodextrin modified HA (host macromer, CD-HA). The ability of the guest and host molecules to interact with their counterpart following conjugation to HA was confirmed by ^1H -NMR spectroscopy and was similar to that of the native complex. Mixing of Ad-HA and CD-HA resulted in rapid formation of a hydrogel composed of guest-host bonds. The hydrogel physical properties including mechanics and flow characteristics were dependent on crosslink density and network structure, which were controlled through macromer concentration, the extent of guest macromer modification, and the molar ratio of guest and host functional groups. The guest-host assembly mechanism permitted both shear-thinning behavior for ease of injection and near-instantaneous reassembly for material retention at the target sight. The hydrogel erosion and release of a model biomolecule were also dependent on design parameters and were sustained for over 60 days. These hydrogels show potential as a minimally-invasive injectable hydrogel for biomedical applications.

Keywords

Supramolecular Assembly; Guest-Host Chemistry; Shear-thinning; Drug Delivery; Hydrogel

INTRODUCTION

Injectable hydrogels continue to gain interest as scaffolds for biomolecule delivery and tissue engineering due to their potential for minimally invasive delivery and tunability in properties.¹⁻³ Many injectable systems have been designed which undergo chemical crosslinking *in situ*, ranging from transdermally photoinitiated systems to autonomous redox reactions or Michael-addition crosslinking. However, *in situ* polymerizing systems may be limited by initial diffusion of the polymer from the injection site⁴ and the potential for premature gelation and resulting delivery failure.⁵ Additionally, many of these systems are based on radical initiation and potential issues such as cytocompatibility^{6,7} and protein bioactivity⁸⁻¹⁰ must be considered. In contrast to hydrogels that form *in situ* with covalent crosslinking, hydrogels based on physical crosslinking mechanisms (e.g., ionic and

*To whom correspondence should be addressed. burdick2@seas.upenn.edu, Tel: 215-898-8537.

Supporting Information

Example ^1H -NMR spectra of Ad₂₀-HA and CD₂₀-HA macromers, fit parameters for guest-host complexation, detailed information on hydrogel compositions, and supporting rheological data. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

hydrophobic interactions, chain entanglement) have also been developed. While physical crosslinking naturally leads to less mechanically robust hydrogels, this may be beneficial as the physical associations are also the basis for advantageous shear-thinning and self-healing behavior.^{11, 12} In these systems, the application of shear force breaks the physical crosslinks and creates fluid-like flow, allowing easy injection of the material through a syringe or catheter, including with cells and therapeutics as cargo. Upon cessation of shear, the hydrogel is able to autonomously reassemble at the target site through these same crosslinking mechanisms.

Numerous systems exhibiting shear-thinning properties have been reported.¹¹ Typically, these systems rely on weak interactions to drive assembly of a global architecture, including formation of entangled β -sheets or microcrystalline domains.^{13–16} As a result of the required macrostructural assembly, these hydrogels often exhibit recovery times on the order of minutes to hours when shear is removed.^{11, 17, 18} These long times may limit their application as injectable hydrogels, since the material components or therapeutic cargo may diffuse from the injection site prior to reassembly. In contrast, systems based on the direct interaction of complementary binding motifs have shown improved recovery times and even near-immediate recovery of initial mechanics in some cases.^{19–22} One example of complementary binding is embodied by guest-host chemical interactions, which are composed of two or more chemical species which interact through non-covalent bonds to drive molecular complexation in a defined structural arrangement. Specifically, host macrocycles such as cyclodextrins have hydrophobic interior cavities which have high affinity for specific hydrophobic guest moieties.²³ Several systems employing guest macrocycles for supramolecular assembly have been reported and reviewed.^{12, 23–27} To date, such systems focused largely on the development of nano and microparticulate drug carriers, viscosupplements, triggerable interactions (e.g., light or heat), or the development of applicable network theory to describe rheological behaviors. While there are some examples on the development of supramolecular hydrogels, these structures have been relatively weak mechanically, exhibited rapid erosion and biomolecule release, or have not exhibited tunable network properties.

For many applications of injectable hydrogels, matching the physical properties of the hydrogel and implant site is desirable. Recent studies have highlighted this, as soft injectable materials resulted in reduced mechanical damage as compared to analogous tougher hydrogels when injected for intrarenal drug delivery.²⁸ Beyond the delivery mechanism, the final hydrogel properties are also important as diffusive properties and hydrogel erosion control biomolecule release^{20, 29} and the physical properties may influence encapsulated cell behavior with respect to cellular migration,^{30, 31} proliferation,^{32–35} and differentiation.^{36–39} Thus, it is important to design systems where biophysical properties may be tailored for specific applications and optimal outcomes.

Here, we report the synthesis and characterization of self-assembling hyaluronic acid (HA) hydrogels based on the guest-host interactions of adamantane-modified HA (guest macromer; Ad-HA) and β -cyclodextrin-modified HA (host macromer; CD-HA). HA was chosen as the backbone polymer since it is found abundantly in tissues and has already been used extensively in biomedical applications.⁴⁰ The HA derivatives are therefore expected to provide a highly biocompatible material as adamantane derivatives are widely used in the pharmaceutical industry⁴¹ and cyclodextrins have attained FDA GRAS approval and are readily included in pharmaceutical, cosmetic, and food products.^{42, 43} Moreover, the specific guest-host pair was chosen because it has been widely investigated and is known to have a relatively high association constant on the order of $1 \times 10^5 \text{ M}^{-1}$.²³ As a result of this high affinity, mixing of the two macromer components in aqueous solution resulted in hydrogels

composed of non-covalent bonds. The goal of this study was to investigate the tunability in network properties and structure, as well as features such as molecule release and erosion.

EXPERIMENTAL SECTION

Materials

Sodium hyaluronic acid (HA, 74kDa) was purchased from Lifecore (Chaska, MN). All other chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

HA-TBA Preparation

The sodium salt of HA was dissolved in DI water at 2 wt%, exchanged against Dowex-100 resin, neutralized by tetrabutylammonium hydroxide, and then frozen and lyophilized.⁴⁴

Ad-HA Synthesis

Various functionalizations of Ad-HA were prepared by coupling 1-adamantane acetic acid (Acros Organics) to HA-TBA via esterification. A round bottom flask was charged with HA-TBA (3.0 g, 4.20 mmol repeat units, 1 eq), 1-adamantane acetic acid (2.45 g, 12.59 mmol, 3 eq), and 4-dimethylaminopyridine (0.38 g, 3.15 mmol, 0.75 eq). The vessel was purged by nitrogen, and anhydrous DMSO added via cannulation to afford an approximately 2wt% solution. Once fully dissolved, di-tert-butyl dicarbonate (BOC₂O) was added via syringe (quantity adjusted to afford the desired final adamantane functionalization: 20%: 0.41 eq, 30%: 0.54 eq, 40%: 0.81 eq, 50%: 1.15 eq) and the reaction carried out at 45°C for 24 hours. Purification was performed by dialysis for 3 days against DI water, precipitation in acetone, and further dialysis. The solutions were frozen and lyophilized to afford the final products. Adamantane functionalization of the macromers was quantified by ¹H-NMR (Bruker 360MHz) and determined from integration of the ethyl multiplet of adamantane (δ =1.50–1.85, 12 H) relative to the HA backbone (δ =3.20–4.20, 10 H). The percent of HA repeat units modified were varied from 20%–50% as denoted by the component subscript.

β -CD-HDA Synthesis

Synthesis of 6-(6-aminohexyl)amino-6-deoxy- β -cyclodextrin (β -CD-HDA) was performed by adaptation of similar reported syntheses.^{45–47} β -cyclodextrin (20 g, 17.62 mmol, 1 eq) was suspended in water (125 mL) and cooled to 0°C. p-toluenesulfonyl chloride (TosCl; 4.2 g, 22 mmol, 1.25 eq) was dissolved in minimal acetonitrile (~10 mL) and added dropwise. The reaction was stirred at room temperature for 2 hours. Sodium hydroxide (2.18 g, 53.6 mmol, 3.1 eq) was dissolved in water and added dropwise. The reaction was stirred at room temperature for 30 minutes before the pH was adjusted to 8.5 by addition of solid ammonium chloride. The solution was cooled on ice and the precipitate collected. The crude product was re-precipitated from cold water (3×200mL), washed by acetone (3×50mL) and dried under vacuum to afford the intermediate 6-o-monotosyl-6-deoxy- β -cyclodextrin (5.12 g, 23%) as a white powder. ¹H-NMR (DMSO-d₆) δ = 2.42 (s, 3 H), 3.12–3.80 (m, overlaps with HOD), 4.12–4.40 (m, 6 H), 4.77 (s, 2 H), 4.83 (s, 5 H), 5.60–6.05 (br s, 14 H), 7.43 (d, 2 H), 7.75 (d, 2 H). A round bottom flask was charged with 6-o-monotosyl-6-deoxy- β -cyclodextrin (5 g, 3.88 mmol, 1 eq), 1,6-hexanediamine (HDA; 20 g, 172.24 mmol, 45 eq), and DMF (25 mL). The reaction was carried out under nitrogen at 80°C for 18 hours. The product was precipitated from cold acetone (5×500mL), washed by cold diethyl ether (2×100mL) and dried under vacuum to afford the final product (3.26 g, 68%) as a white powder. ¹H-NMR (DMSO-d₆) δ = 1.14–1.61 (m, 12 H), 3.12–3.45 (m, overlaps with HOD), 3.48–3.78 (m, 28 H), 4.28–4.56 (br s, 6 H), 4.83 (s, 7 H), 5.59–5.88 (br s, 14 H).

CD-HA Synthesis

CD-HA was prepared by coupling β -CD-HDA to HA-TBA via amidation.³⁹ A round bottom flask was charged with HA-TBA (2.5 g, 3.43 mmol repeat units, 1 eq) and β -CD-HDA (2.96 g, 4.86 mmol, 0.5 eq). The vessel was purged by nitrogen and anhydrous DMSO added via cannulation to afford an approximately 2wt% solution. Once fully dissolved, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP; 1.06 g, 4.86 mmol, 0.5 eq) was dissolved in minimal DMSO and added via syringe. The reaction was carried out at room temperature for 2 hours. Purification was performed by extensive dialysis against DI water and filtration to remove insoluble byproducts from the reaction. The solution was frozen and lyophilized to afford the final product. Functionalization was determined from integration of the hexane linker (δ =1.35–1.85, 12 H) relative to the methyl singlet of HA (δ =2.1, 3 H). The percent of HA repeat units modified was ~20% as denoted by the component subscript.

Determination of Apparent Binding Affinity

To achieve the required solubility, 1-adamantane acetic acid was converted to the sodium salt by neutralization with sodium hydroxide. Either the resulting 1-adamantane acetate sodium salt or Ad₂₀-HA was dissolved at approximately 2.5mM in DI water and titrated by either β -cyclodextrin or CD-HA. Samples were frozen, lyophilized, and reconstituted in D₂O for ¹H-NMR spectroscopy. The ethyl chemical shift of adamantane (CH₂, 6H, δ_0 =1.61) was used to monitor guest-host complex formation. The apparent binding affinity (K_a) was determined by fitting the change in this shift ($\Delta\delta$) and total β -cyclodextrin concentration $[H]_0$ to the Benesi-Hildebrand equation

$$\frac{1}{\Delta\delta} = \frac{1}{K_a} \frac{1}{\Delta\delta_{max}[H]_0} + \frac{1}{\Delta\delta_{max}}$$

where $\Delta\delta_{max}$ is the maximum possible change in this NMR shift occurring with a large excess of β -cyclodextrin, extrapolated from the curve fit. Linear models for each case were fit in R and compared by ANOVA.

Hydrogel Formation

For all characterization studies, hydrogels were prepared from stock solutions of the individual macromers in PBS at the desired concentration. For hydrogel formation by the guest-host assembly mechanism, the two component solutions were combined and mixed by manually stirring to ensure a homogenous hydrogel which was then briefly centrifuged to remove entrapped air. Unless otherwise stated, adamantane and β -cyclodextrin were present in stoichiometric balance, and the concentration refers to the overall weight percent of combined macromers.

Rheological Characterization

All characterization was performed using an AR2000 stress-controlled rheometer (TA Instruments) fitted with a 20 mm diameter cone and plate geometry, 59 min 42 s (0.995°) cone angle, and 27 μ m gap. Rheological properties were examined at 25°C by oscillatory frequency sweeps (0.01–100 Hz; 1% strain), oscillatory time sweeps at various frequencies (0.01, 1.0, 10, or 100 Hz; 1% strain), oscillatory strain sweeps (0.01–500% strain 10Hz), and continuous flow experiments with the shear rate linearly ramped from 0–0.5 s⁻¹ or 0–5.0 s⁻¹ and returned. For shear recovery experiments, shear-thinning was performed at 250% strain with recovery at 0.5% strain, each at 10 Hz. Frequency and time sweeps were performed on a minimum of 3 different samples and comparisons of moduli, loss tangent, and bulk

relaxation times between groups were performed by an unpaired Student's t-test. Comparison within groups at varying frequency was performed by a paired Student's t-test.

Fluorescent Labeling

For erosion studies, Ad_x-HA products were fluorescently labeled to allow direct quantification of polymer concentrations in solution. Following adamantane modification, products were again converted to their corresponding tetrabutylammonium salts. Alexa Fluor 350 hydrazide was then coupled by BOP mediated coupling in DMSO and purification was performed by extensive dialysis.

Hydrogel Erosion and BSA Release

Custom fabricated molds of cast acrylic plastic (McMaster-Carr) were used to contain the hydrogels within a 5 mm diameter and 6 mm high depression exposed directly to an overlying reservoir of buffer. The hydrogels were either fluorescently labeled for erosion or loaded with 0.1 wt% fluorescein isothiocyanate conjugated bovine serum albumin (FITC-BSA) as a model biomolecule to monitor release behavior. Hydrogels were prepared as above, loaded into a 0.5cc syringe, and 20 μ L of the desired hydrogel was injected into the depression. The mold was briefly centrifuged to level the hydrogel surface. Hydrogels were covered with 1 mL PBS and allowed to erode for up to 60 days statically at 25°C. At specified time points, the buffer was removed from the well and replaced with fresh buffer. Macromer and BSA release were quantified calorimetrically relative to standard curves, and data points represent the mean and standard deviation of three gels for each composition.

RESULTS AND DISCUSSION

Chemical Modification of HA

The controlled esterification of HA with carboxylic acid containing groups has been previously demonstrated to be a powerful means of reproducibly controlling HA functionalization.⁴⁸ Similar methodology was utilized here (Scheme 1A) to couple adamantane to HA, where 1-adamantane acetic acid was present in excess and the final HA functionalization was controlled simply by altering the molar ratio of BOC₂O to HA repeat units. The chemical structure of Ad-HA was confirmed by ¹H-NMR spectra (Figure S1), and functionalizations of 21.0, 29.1, 39.2, and 49.8% were produced as confirmed by integration of the ethyl multiplet of adamantane (δ =1.50–1.85, 12 H) relative to the HA polymer backbone (δ =3.20–4.20, 10 H).

Various methods of producing CD grafted HA have been previously reported, including the use of the Mitsunobu reaction and coupling through reductive amination.^{49, 50} Here, we utilized the amine coupling of β -CD-HDA to HA (Scheme 1A), as the methodology allows for homogenous reaction conditions, controlled final functionalizations, formation of a stable amide linkage, and requires a monofunctionalized derivative of β -cyclodextrin which is easily prepared (Scheme 1B). The chemical structure of CD-HA was confirmed by ¹H-NMR (Figure S2) and a functionalization of 20.6% was determined by integration of the hexane linker (δ =1.35–1.85, 12 H) relative to the methyl singlet of HA (δ =2.1, 3 H).

Inclusion Ability of Guest and Host Macromers

The hydrogel crosslinking scheme used here is based on the specific and direct interaction of two complementary binding motifs, namely the guest-host complexation of adamantane and β -cyclodextrin. As discussed in a recent review, others have reported inhibition of the CD complex formation with guest compounds having a low molecular weight or molar volume when the guest is attached to a polymer chain.⁴³ It is therefore essential to the molecular

design that the binding of the guest and host motifs is not impeded, such as by steric hindrance of the proximal bulky HA polymer backbone.

The apparent binding affinity of the native guest-host complex and the synthesized macromers was examined, as depicted in Figure 1A. For each case, the guest component was dissolved and titrated by the corresponding host component. As a result of the hydrophobic interaction within the guest-host complex, a pronounced down-field shift and broadening of the ethyl peak was observed in the ^1H -NMR spectra of adamantane (Figure 1B). This shift was monitored throughout the titration and utilized to construct mole ratio plots of the chemical shifts due to this interaction. All groups show a strong linear correlation between the chemical shift and mole fraction ($R^2 > 0.98$, Figure 1C), indicative of the known one-to-one association of adamantane with β -cyclodextrin.

The apparent binding affinity was determined for each system by fit to the Benesi-Hildebrand equation. Fit parameters and apparent association constants are summarized in Table S1. While large error estimates in the maximum shift make it difficult to directly compare the association constants reliably,⁵¹ no significant increase in slope for the linear models (i.e. decrease in binding affinity) was observed for the grafted guest and host molecules relative to the native complex ($p > 0.054$). The association constants are similar for all cases examined, ranging from 503 to 536 M^{-1} and on the order of those determined by similar spectrographic methods.⁵² The results confirm that the chosen macromer design follows the expected one-to-one guest-host complexation and that tethering of the guest or host molecule to HA does not impede the binding of these components with their complement.

Hydrogel Formation

When HA macromers modified with either adamantane or cyclodextrin were mixed in solution, near-immediate formation of a pseudoplastic hydrogel occurred through the complexation of reversible guest-host moieties on complementary macromers (Figure 2A). Specifically, while the individual macromer solutions were flowable liquids, the formed hydrogel was stable to a qualitative inversion test (Figure 2B). Oscillatory rheology confirmed that individual macromers were both viscous solutions and that an increase in moduli of several orders of magnitude occurred following mixing (Figure 2C).

The utility of spectroscopic methods previously discussed are limited to solutions, as hydrogel formation results in freezing of the NMR signal. Thus, the interaction of the Ad-HA and CD-HA in a one-to-one fashion was confirmed by oscillatory rheology of hydrogels (7.5 wt%) consisting of either excess Ad₂₀-HA or CD₂₀-HA. Representative frequency sweeps (Figure S3A) demonstrate a decrease in relaxation times and moduli for hydrogels containing excess Ad₂₀-HA. Further quantification (Figure S3B) of the storage and loss moduli at various stoichiometries shows a clear convergence on maximal properties when the guest and host are present in a one-to-one ratio, confirming that balanced stoichiometry affords the most robust hydrogel. As highlighted in Table S2, this condition also coincides with the maximum theoretical crosslink density as may be expected for such self-assembling systems.

Control of Network Properties

The crosslink density and architecture may be varied by the concentration of the individual macromers and the extent of functionalization of the macromers with either the guest or host molecules, as well as the ratio of guest to host components. It has been previously proposed that two primary junction points exist within binary associating systems similar to those developed here, as depicted in Figure 3A. These include both weak network junctions

composed of few guest-host complexes and multifold junctions where multiple polymer chains entangle and are joined by several guest-host complexes.⁵³ Multifold junctions contribute largely to the properties of the hydrogels, as the sum of many guest-host interactions results in an avidity greater than the affinity of a single guest-host bond. While both of these junction point architectures may contribute to the moduli, the multifold junctions dominate the slow dynamic properties of the hydrogel as stress relaxation at these junctions requires the simultaneous slipping of multiple guest-host bonds. For any binary associating system, these two types of junction points must exist in a thermodynamic equilibrium which governs the time-dependent properties of the network.²⁷

In order to control the properties of assembled hydrogels, we altered the crosslink density and junction point architecture through both the macromer concentration and the guest macromer modification. First, an increase in the macromer concentration (2.5 to 10 wt%) was utilized to increase the theoretical crosslink density from 4.87 to 21.13 mM. At concentrations of 2.5 wt% and below, hydrogels were observed to assemble and exclude excess solvent, which prevented consistent rheological measurement; at concentrations greater than 10 wt%, solutions were highly viscous and homogenous mixing was difficult. Separately, the guest macromer modification was increased from 20 to 50% while maintaining a macromer concentration of 7.5 wt%. Although this resulted in a minimal change in the theoretical crosslink density (15.42 to 19.97 mM), the proportion of effective multifold junctions is expected to increase as formation of guest-host complexes requires the coordination of multiple polymer chains. The frequency response of the materials illustrates the changes in network structure for these separate conditions. Specifically, while an increase in the macromer concentration resulted in an increase in moduli as well as a moderate reduction in crossover frequency (Figure 3B), an increase in the guest macromer modification resulted in drastic changes in the crossover frequency with little change in the moduli across groups (Figure 3C).

Quantification of these observations across the frequency range demonstrated the hypothesized phenomena. Notably, the storage modulus increased with both macromer concentration and frequency according to a power law (Figure 4A, S4A). Similar relationships were observed for the loss moduli (Figure S4A), though a more rigid solid was formed with increasing macromer concentration as indicated by the decrease in the loss tangent ($\tan(\delta)$, Figure 4C, S5A). However, the moduli of hydrogels composed of varying guest macromer modifications did not scale similarly with frequency. Rather, a moderate increase in moduli was observed across groups, which was significant only at low frequencies (Figure 4B, S4B). A great reduction in the loss tangent (Figure 4D, S5B) at high modifications and low frequencies suggests the change in properties is a result of the network junction architecture. In particular, a disproportionate increase in the proportion of multifold junctions must limit network relaxation and thereby increase moduli at low frequency.

To corroborate these observations, the bulk relaxation time was examined. The relaxation time is the characteristic timescale over which an applied stress is dissipated by viscous behavior of the pseudoplastic hydrogel (i.e. bond re-organization to eliminate internal shear stress) and is directly determined from the loss tangent as a function of frequency ($\tan(\delta)=1$; see Figure S6). Guest macromer modification was found to exhibit a greater influence over the bulk relaxation time than macromer concentration (Figure 5A, B). For instance, an increase in polymer concentration from 7.5 to 10 wt% resulted in a four-fold increase in the bulk relaxation time; whereas, a similar increase in the theoretical crosslink density by increasing macromer modification from 20 to 50% resulted in a nearly ninety-fold increase in the bulk relaxation time. These results support the notion that an increase in the proportion of multi-fold junctions is produced by a mismatch of the guest and host

macromer modifications, and the effects on hydrogel behavior are visually demonstrated in long-term flow experiments (Figure 5C). It was observed that hydrogels having relatively short bulk relaxation times flow to allow relaxation of internal shear stresses. However, hydrogels consisting of guest macromers with high modifications exhibited negligible flow over the course of one week. Taken together, the results demonstrate a methodology to independently control the moduli and relaxation behavior of binary associating systems.

Shear-thinning and Recovery

Since the guest-host assembly mechanism is based on reversible dynamic bonds, formed hydrogels exhibited flow and recovery characteristics. To demonstrate the desired shear-thinning ability required for injectable delivery systems, hydrogels were subject to continuous flow experiments. Here, the shear rate was linearly ramped from 0 – 0.5s^{-1} to investigate the effect of flow rate on viscosity and resultant shear stress. In this regime of simulated slow flow conditions, hydrogels that were formed from various macromer concentrations (Figure 6A) or extent of guest macromer modification (Figure 6B) show the desired shear-thinning behavior where viscosity decreases with shear rate. Consequently, the shear stress approached a maximum. The terminal viscosity and shear stress magnitudes were observed to increase with both macromer concentration and modification of the guest macromer, while the profiles of shear stress and viscosity changes with increased shear rate were similar across all systems investigated.

While this behavior demonstrates the shear-thinning ability of this hydrogel system, the shear rate experienced within a syringe may be far greater than those explored here.⁵⁴ Upon exploring higher shear-rates (up to 5s^{-1}), trends in shear-thinning behavior were not observed to change for increases in macromer concentration; however, an increase in the guest macromer modification resulted in a pronounced decline in shear-stress at high shear-rates (Figure S7). Similar trends have been examined in various supramolecular networks, and shown to be a consequence of network rearrangement during shear-induced flow.^{12, 27, 55, 56} Specifically, if the chain relaxation time is slower than the reconnection of unassociated crosslinks, then a regime of drastic shear-thinning may be induced above a critical shear rate. From these observations and previously discussed bulk relaxation behavior, it is reasonable to conclude that the increased local stability of multifold junctions decreases the chain relaxation time, resulting in pronounced shear-thinning behavior when the extent of guest macromer modification is increased. For biomedical applications, such drastic shear-thinning behavior may be advantageous as it increases the materials ability to flow through a catheter or narrow gauge syringe, which reduces the necessary force for injection.

To examine the timescale of material recovery, such as following injection, hydrogels were subjected to cycles of large amplitude oscillatory strain followed by low amplitude oscillatory strain. First, a strain sweep (Figure 6C) was used to determine the yield stress for the hydrogel examined ($\sim 60\%$ strain at yield), from which a low (0.5%) and high (250%) strain were determined for recovery experiments. Under cyclic deformation, the materials demonstrated a clear decline in moduli and concurrent gel-sol transition at the onset of high strain conditions (Figure 6D). At the transition from high to low strain conditions, rapid recovery to initial mechanics was observed. Even at a sampling rate of 0.75Hz , the transition could not be observed as greater than 80% of the initial moduli were recovered in this time and complete recovery was observed within 30 s . Similar recovery characteristics were observed regardless of the number of cycles performed or the composition of the hydrogel. These results indicate that the materials are capable of near-immediate recovery following shear-thinning delivery, allowing optimal retention of both material components and potential therapeutic cargo at the target site.

Hydrogel Erosion and Biomolecule Release

Hydrogel erosion and the sustained delivery of therapeutic biomolecules are required for many applications and the desired degradation and release kinetics are dependent on the specific application. Although this study is not targeted towards a single application, we investigated the tunability of hydrogel erosion and concurrent biomolecule release through changes in crosslink density and network architecture. Hydrogels investigated were contained within the depression of custom fabricated erosion cells and exposed directly to buffer. For controls comprised of individual macromer components, near immediate dissolution into the buffer was observed. For all hydrogels investigated, initial high erosion rates were followed by a slower phase of nearly linear erosion (Figure 7A, B). The initial release of macromer is likely related to macromers that are not well incorporated to the bulk system and can be released rapidly as the dynamic guest-host interactions become limited. Increasing the crosslink density through the macromer concentration resulted in a clear trend of decreased erosion rates (Figure 7A). This is expected as the disassembly of gels is related to the number of guest-host interactions in the network. Interestingly, increases in the guest macromer modification were found to increase the rate of erosion, particularly in the later near linear phase (Figure 7B). These results are counterintuitive considering the erosion of dynamically bonded hydrogels are known to be dependent on valency of the associating groups, as erosion of a macromer requires the simultaneous dissociation of all binding groups.²⁰ In this case, erosion likely proceeds by surface erosion of small clusters of the macromers which are no longer linked to the bulk hydrogel, suggesting network homogeneity plays an important role in long-term stability.

As cyclodextrins are known to interact strongly with a great variety of small organic molecules, primarily through hydrophobic interactions, they have been extensively employed in the pharmaceutical industry. Particularly, cyclodextrins have been investigated for their ability to complex with and control the release of small organic molecules,^{24, 26} and their interaction with proteins has also been investigated for pharmaceutical applications as discussed in recent reviews.⁴³ While such small molecule interactions have proven efficacy, the utilization of supramolecular network structure to control biomolecule release has rarely been examined.⁵⁷ In order to perform a generalizable examination of this behavior, we utilize bovine serum albumin (BSA) as it is a moderately sized biomolecule (66.5 kDa) with globular structure. Some proteins may contain phenylalanine or tyrosine residues on the surface, enabling binding of cyclodextrins to the protein and resulting in interference with release. It is known that no such significant interaction exists between β -cyclodextrin and BSA⁵⁸ and the utilization of FITC-BSA has previously been scrutinized in release from β -cyclodextrin based materials and no interaction with fluorescein was observed.⁵⁹

In this general study, we observed sustained release that was dependent on both the macromer concentration and guest macromer modification. For all hydrogels examined, release beyond 60 days was observed, which extends the release capabilities beyond what has been achieved in hydrogels based on similar supramolecular crosslinking. Importantly, release rates were readily tailorable according to the macromer concentration (Figure 7C). Specifically, the time until 90% cumulative release was increased from approximately 21 to 53 days between 2.5 and 10 wt% hydrogels. While trends in protein release followed those of the erosion profiles, protein release occurred faster than erosion, indicating that a combination of diffusion and surface erosion likely contributes to release. This is in contrast to similar reported systems employing high polymer concentrations (>20 wt%) that showed zero-order release consistent with primarily surface erosion.⁵⁹ The effect of surface erosion is apparent in the release from hydrogels of various guest macromer modifications. Here, a tendency towards increased release rates with guest macromer modifications was observed, despite a moderate increase in crosslink density (Figure 7D). These observations are consistent with the previously described increase in erosion for these hydrogels. The

influence of this erosion based release mechanism minimally affected release, with the time to 90% cumulative release occurring between 39 and 46 days for all hydrogels within this group. The observations herein provide a strong basis of understanding the design parameters controlling hydrogel erosion and biomolecule release for the guest-host hydrogels developed, perpetuating their use in future applications.

CONCLUSION

Self-assembling HA hydrogels based on a guest-host assembly mechanism were developed. The reactions chosen for modification of HA with the host cyclodextrin and guest adamantane were simple, direct, and resulted in the desired products with controlled functionalization and potential for scalable synthesis. The resulting guest and host macromers were found to have a binding capacity which was unhindered by the molecular structure. Formation of a hydrogel by simple mixing of the two macromer components in aqueous solutions was demonstrated, and the properties of the resulting hydrogel was dependent on crosslink density and network architecture which were separately controlled by macromer concentration and guest macromer modification. Importantly, all hydrogel formulations examined were capable of shear-thinning behavior to enable ease of injection as well as rapid recovery to localize the hydrogel and its potential therapeutic cargo at the site of injection. Erosion and model biomolecule release demonstrated a remarkable stability and tunability based on hydrogel design. In short, the hydrogel system developed shows great potential as a minimally-invasive injectable material platform due to its demonstrated tunable mechanical properties, near-ideal flow and recovery characteristics, and remarkable stability towards erosion which affords controlled long-term biomolecule release.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Confirmation of guest-host complexation for modified macromers by titration of the guest (adamantane, Ad) by the host (β -cyclodextrin, CD). (A) Schematic of Ad and CD interactions examined, either alone or when either Ad or CD are coupled to HA (illustrated as black line). (B) Corresponding ^1H -NMR spectra of Ad monitored throughout the titrations. (C) Mole ratio plots showing the ethyl chemical shift of Ad with various molar ratios of CD/Ad investigated.

Figure 2.

Overview of hydrogel formation. (A) Schematic of dynamic crosslink formation utilizing guest-host complexation. (B) Qualitative inversion test; $Ad_{20}HA$ 5wt% (blue, left), $CD_{20}HA$ 5wt% (red, middle), $CD_{20}HA + Ad_{20}HA$ 5wt% (purple, right). (C) Oscillatory time sweeps of individual macromers and hydrogel formed at 5wt%; storage modulus (G' , filled symbols) and loss modulus (G'' , empty symbols) at 10 Hz, 1.0% strain.

Figure 3.

Example frequency dependence of material behavior. Schematic representation of a weak network junction (top) and a multifold junction (bottom) that are proposed to constitute the network junction points (A). Representative oscillatory frequency sweeps showing storage modulus (G' , filled symbols) and loss modulus (G'' , open symbols) of hydrogels composed of different macromer concentrations (B) or Ad_x -HA modifications (C) as indicated.

Figure 4.

Frequency dependence of material properties. Storage modulus (G') of hydrogels composed of different macromer concentrations (A) or Ad_x-HA modifications (B). Loss tangent ($\tan(\delta)$) of hydrogels composed of different macromer concentrations (C) or Ad_x-HA modifications (D). Values represent the mean measurement of 3 different hydrogels. Please note the directional change in frequency magnitude between the storage modulus and loss tangent plots. For two-dimensional representation, including loss moduli and statistical analysis, please refer to Figure S4 and S5.

Figure 5.

Bulk relaxation behavior of hydrogels. Relaxation times with varied macromer concentration (A) or Ad_xHA modification (B). Values were quantified from the frequency sweeps of three independent hydrogels and represent the mean $\pm$ standard deviation of a minimum of 3 samples. All groups are significantly different with the exception of 40 to 50% Ad_xHA modification. (C) Images of hydrogels of varied composition that were formed in the bottom of glass vials and the vials were placed on their sides for up to one week.

Figure 6.

Guest-host hydrogel flow and recovery characteristics. Continuous flow experiments showing the shear stress (closed symbols) and viscosity (open symbols) of hydrogels composed of different macromer concentrations (A) or $\text{Ad}_x\text{-HA}$ modifications (B) as indicated by adjacent labels. Storage modulus (G' , red dotted line), loss modulus (G'' , blue dashed line), and loss tangent ($\tan(\delta)$, black solid line) of a hydrogel undergoing a strain sweep at (C) or cyclic deformation (D) of 0.5% (low, unshaded areas) and 250% (high, shaded areas) strain at 10Hz.

Figure 7.

Erosion and molecule release characteristics. Cumulative guest-host hydrogel erosion profiles for various macromer concentrations (A) or Ad_x -HA modifications (B). Model biomolecule (FITC-BSA) release from hydrogels composed of different macromer concentrations (C) or Ad_x -HA modifications (D). Values represent the mean $\pm$ standard deviation of 3 different hydrogels, each initially loaded with 0.1wt% FITC-BSA.

Scheme 1.

Synthesis of guest ($\text{Ad}_x\text{-HA}$) and host ($\text{CD}_{20}\text{-HA}$) macromers.

(A). (i) 3.0 equiv Ad-COOH , DMAP/BOC₂O, DMSO, 45°C, 24 h. (ii) 0.5 equiv $\beta\text{-CD-HAD}$, 0.5 equiv BOP, DMSO, room temp, 2 h. (B). (i) 1.25 equiv TosCl , NaOH, H_2O , room temp, 30 min. (ii) 45 equiv HDA , DMF, 80°C, 18 h.