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Sustained Small Molecule Delivery from Injectable Hyaluronic Acid Hydrogels through Host-Guest Mediated Retention

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Abstract

Self-assembled and injectable hydrogels have many beneficial properties for the local delivery of therapeutics; however, challenges still exist in the sustained release of small molecules from these highly hydrated networks. Host-guest chemistry between cyclodextrin and adamantane has been used to create supramolecular hydrogels from modified polymers. Beyond assembly, this chemistry may also provide increased drug retention and sustained release through the formation of inclusion complexes between drugs and cyclodextrin. Here, we engineered a two-component system from adamantane-modified and β-cyclodextrin (CD)-modified hyaluronic acid (HA), a natural component of the extracellular matrix, to produce hydrogels that are both injectable and able to sustain the release of small molecules. The conjugation of cyclodextrin to HA dramatically altered its affinity for hydrophobic small molecules, such as tryptophan. This interaction led to lower molecule diffusivity and the release of small molecules for up to 21 days with release profiles dependent on CD concentration and drug-CD affinity. There was significant attenuation of release from the supramolecular hydrogels (~20% release in 24h) when compared to hydrogels without CD (~90% release in 24h). The loading of small molecules also had no effect on hydrogel mechanics or self-assembly properties. Finally, to illustrate this controlled delivery approach with clinically used small molecule pharmaceuticals, we sustained the release of two widely used drugs (i.e., doxycycline and doxorubicin) from these hydrogels.

Keywords

self-assembly; small molecules; sustained release; host-guest chemistry; hydrogel; drug delivery; cyclodextrin

Introduction

Towards the development of translational, multifaceted strategies for treating disease, injectable hydrogels provide unique properties to deliver therapeutics (e.g., cells, drugs) to tissues. Self-assembly, driven through non-covalent interactions, may impart shear-thinning and self-healing properties to hydrogels, which allows direct injection into tissues. A number of non-covalent interactions have been investigated to form self-assembled

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hydrogels, including hydrophobic interactions or host-guest assembly of macrocycles such as cucurbiturils and cyclodextrins (CDs).[1-3] The association between polymer chains leads to the self-assembly of the network, which can disassemble under shear (shear-thinning) to permit flow. When the shear force is removed, the material can associate again (self-healing). These properties are dependent on the specific association between the chemistry involved in polymer assembly.

CDs are cyclic macromolecules formed from 6, 7, or 8 α-D-glucopyranoside units, termed α-, β-, and γ-CD respectively. As a strategy for self-assembly, these molecular interactions have been employed to form hydrogels between polymers modified with β-CD and adamantane (Ad), α-CD and PEG, and β-CD and azobenzene, among other polymer systems.[3-5] These self-assembled hydrogels have been used towards a number of biomedical therapies, including mechanical interventions, cell delivery, and sustained therapeutic release.[6-9] CD host-guest chemistry has previously been used in the design of drug delivery systems. Notably, the Davis group has developed cationic CD polymers that ionically complex with nucleic acids to form nanoparticles, that may be functionalized with targeting ligands via Ad interactions to promote receptor-mediated endocytosis.[10] Regarding sustained release, self-assembled hydrogels provide easily injectable depots of therapeutics; however, their investigation as drug delivery systems has largely been focused on macromolecules where release is governed through network properties. Despite recent advances in supramolecular systems, few supramolecular hydrogels have been investigated for small molecule release, due to difficulties associated with using these traditional mechanisms (e.g. matrix erosion, control of mesh size) to sustain the release of small molecules.

Small molecule therapeutics are potent drugs that can be used in such strategies as chemotherapy or the inhibition of pathologic matrix metalloproteinase (MMP) activity, where there is a need for systems that permit localized and sustained therapeutic delivery.[11] Due to the highly hydrated nature of hydrogels and their large mesh size, most small molecules will diffuse out rapidly in a matter of hours, which is often too fast to provide sustained therapeutic effects. To address this challenge, strategies using composite systems of hydrogels and hydrophobic micro/nanoparticles or covalent conjugation of drugs to polymer backbones have been developed to provide sustained small molecule release.[12] While these strategies are effective, they require added complexity in hierarchical formulation or offer regulatory translational limitations by modifying drug molecules. A third strategy that may be used to sustain small molecule release is to engineer non-covalent affinity between small molecules and polymers to increase retention time. Hydrogels using affinity mediated sustained release have recently been developed leveraging interactions between therapeutic peptides or hydrophobic molecules and polypeptide-based hydrogels, as well as between macrocycle hosts and guest ligands.[7, 13, 14]

CDs have remarkable affinity for a wide variety of small molecule guests, which has led to the use of CDs as excipients in a number of small molecule pharmaceuticals.[15, 16] In addition, these host-guest interactions have been leveraged to increase molecule retention within covalently crosslinked hydrogels.[14, 17-20] In one example, antibiotic release was sustained for ~10 days from covalently crosslinked polyurethane hydrogels of β-CD and

multivalent isocyanates that were used to coat metal screws and polymer meshes.[14, 21] β-CD also forms inclusion complexes with a wide variety of other molecules, including chemotherapeutics such as doxorubicin, MMP inhibitors such as doxycycline, and even aromatic amino acids such as tryptophan.[22-26] However, β-CD inclusion complexes have not yet been leveraged to provide sustained small molecule release from hydrogels formed via supramolecular self-assembly.

Recently, our group developed a two component injectable hydrogel formed from hyaluronic acid (HA) modified with Ad (Ad-HA) and HA modified with β-CD (CD-HA).[3] HA is a natural component of the extracellular matrix, which interacts with cell surface receptors and can even provide stem cell recruitment.[27, 28] We hypothesized that alterations in CD content and payload affinity for CD would alter the release of molecules from hydrogels (Figure 1). Such a system combines the unique shear-thinning and self-healing properties previously reported in these hydrogels for injectability with the unique host-guest inclusion capability of β-CD for a number of small molecule drugs. We believe this to be the first example of an injectable hydrogel that has such functionality.

Experimental

Materials

Sodium hyaluronic acid (HA, 90kDa) was purchased from Lifecore (Chaska, MN). β-cyclodextrin (CD), 1-adamantane acetic acid, and hexanediamine (HDA) were purchased from TCI America (Portland, OR). Fluorenylmethyloxycarbonyl (FMOC) protected amino acids and (1H-Benzotriazol-1-yloxy)(dimethylamino)-N,N-dimethylmethaniminium hexafluorophosphate (HBTU) were purchased from Novabiochem (Billerica, MA). All other materials were purchased from Sigma-Aldrich (St Louis, MO).

Macromer Synthesis

Ad-HA was synthesized according to previously reported protocols.[3] Briefly, HA was first converted to a tetrabutylammonium salt (HA-TBA) through the use of an ion exchange resin (Dowex 50Wx4). HA was dissolved in deionized (DI) water (resistivity 19.6 MΩ cm) at 3 wt% and mixed with the resin for 4 h to allow for ion exchange. The resulting solution was filtered, neutralized with tetrabutylammonium hydroxide, frozen, and lyophilized to produce the HA-TBA salt. To synthesize Ad-HA, HA-TBA, 4-methylaminepyridine, di-tertbutyl dicarbonate and Ad were then dissolved using dimethyl sulfoxide (DMSO) and allowed to react for 20 h at 45 °C under nitrogen. The resulting Ad-HA product was then purified by dialysis against DI water for 3 days, precipitated in acetone, and then further dialyzed against DI water for a total of 2 weeks. The aqueous polymer solution was then frozen, lyophilized, and analyzed for purity and percent modification using ^{1}H-NMR (DMX 360, Bruker, Billerica, MA). All Ad-HA used in this study had ~25 % of HA repeat units modified with Ad groups (Figure S1).

To facilitate conjugation to HA, β-CD was aminated using an HDA linker. First, β-CD was tosylated through the reaction of *p*-toluenesulfonyl chloride and β-CD in aqueous solution. Sodium hydroxide (3 mol NaOH/ mol CD) was added dropwise to the solution and stirred for 30 min at room temperature. Next, ammonium chloride was added to adjust the pH to

~8.5 and precipitate the β-CD tosylate. β-CD tosylate was repeatedly washed in water and acetone and then dried under vacuum to use in the amination reaction. A 2-neck flask was charged with β-CD tosylate, HDA, and DMF. The flask was connected to a condenser, purged by nitrogen, and reacted for 16 h at 80 °C. CD-HDA was purified by repeated washes with acetone and diethyl ether to yield aminated β-CD (CD-HDA). To form CD-HA, HA-TBA, CD-HDA, and (benzotriazol-1-ylozy)tris(dimethylamino)phosphonium hexafluorophosphate were dissolved in DMSO in a round bottom flask. The reaction was allowed to proceed for 3 h at room temperature. CD-HA was then purified by dialysis against water for 2 weeks, frozen, lyophilized, and characterized using ^{1}H-NMR. CD-HA used in this study was modified at ~35 % of HA repeat units for calorimetry experiments and ~25 % for all other experiments (Figure S1).

Methacrylated HA (MeHA) was synthesized according to previously reported protocols.[29] Briefly, sodium HA was dissolved in DI water and methacrylic anhydride was added incrementally to the solution, while maintaining the pH between 7.5 - 8.5 for 5 h. The solution was then dialyzed extensively against DI water, frozen, lyophilized, and characterized using ^{1}H-NMR. MeHA used in this study was modified at ~30 % of HA repeat units (Figure S1).

Peptide Synthesis

Peptides were synthesized using standard solid phase synthesis on a solid phase peptide synthesizer (PS3, Protein Technologies Inc., Tuscon, AZ). Two fluorescein modified peptides, GKWEWKWE-FITC (3W) and GKGEGKGE-FITC (NoW) were synthesized as model drugs that have a high or low affinity for β-CD, respectively. Peptides were synthesized on a glycinol 2-chlorotrityl resin and with FMOC protected amino acids. Peptides were cleaved in a mixture of trifluoroacetic acid, triisopropylsilane, and water (95:2.5:2.5) and then purified through precipitation in cold diethylether. Solid peptides were dissolved in DI water, frozen, and lyophilized. Peptide purity and sequence were confirmed using MALDI-TOF spectroscopy (Multiflex, Bruker, Billerica, MA). Masses for both 3W (expected 1549.6, m/z 1571.8, corresponding to a complex with Na ion) and NoW (expected 1162.1, m/z 1162.6) confirmed successful peptide synthesis.

Hydrogel Preparation

Hydrogels were prepared by first dissolving CD-HA and Ad-HA in Dulbecco's phosphate buffered saline (PBS) with ionic content of 137 mM NaCl, 2.67 mM KCl, 1.47 mM KH_2PO_4, 8.05 mM Na_2HPO_4 or PBS containing drug overnight at room temperature (21 °C). The CD-HA and Ad-HA solutions were mixed to induce gelation. For notation, hydrogels are referred to as x:y (z wt%) hydrogels, with x:y referring to the Ad:CD stoichiometric ratio within a given hydrogel, and z wt% referring to the total macromer weight percent of the hydrogel. All guest-host hydrogels were made using 25 % modified CD-HA and 25 % modified Ad-HA.

MeHA hydrogels were formed through a photoinitiated radical polymerization of a macromer solution. MeHA at 7.8 wt% macromer concentration was dissolved in PBS containing fluorescent peptides and 0.05 wt% I2959 photoinitiator. MeHA macromer

solution was then stored under nitrogen and polymerized using UV light at 10 mW/cm^2 (Omnicure S1000, Lumen Dynamics, Mississauga, Ontario, Canada) for 5 min to induce gelation.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed using a low volume isothermal titration calorimeter (Microcal iTC200, Worcestershire, UK). Briefly, aqueous polymer solutions at 1 mg/mL and 0.468 mg/mL for CD-HA and HA, respectively, were titrated in 2 μL aliquots to a thermal cell filled with 200 μL of tryptophan at 40 μM. Differential power was recorded between the sample cell and a reference cell containing MiliQ water at a constant temperature of 25 °C. Baseline titrations of polymer solution into MilliQ water were subtracted in order to remove heats of dilution associated with polymer solution injection. The resulting isotherms were analyzed with Origin 7 software according to a one-binding site fit to determine association constants. In order to reduce errors associated with fitting multiple parameters (association constant, enthalpy, and binding stoichiometry), stoichiometry was pre-defined according to CD modification determined through ^{1}H-NMR.

Rheology and Degradation

Hydrogel mechanics were assessed using a stress-controlled rheometer (AR2000, TA Instruments, New Castle, DE) fitted with a 20 mm diameter cone (0.995° angle) and plate geometry having a 27 μm gap. Oscillatory time sweeps were conducted at 0.1, 1, and 10 Hz at 1% strain. Additionally, oscillatory frequency sweeps were performed between 0.01 Hz and 100 Hz at 1 % strain. Finally, to simulate injection, hydrogels underwent a cycle of strain between 1 % (low strain) and 500 % (high strain) at 1 Hz. Storage and loss moduli were analyzed for both drug loaded and unloaded hydrogels.

Hydrogel degradation was assessed using a colorimetric assay for hyaluronic acid. Briefly, 30 μL of sample collected from erosion cells was degraded in 1 mL of concentrated sulfuric acid at 100 °C for 10 minutes. Next, 30 μL of 0.125% carbazole in ethanol was added to each sample, vortexed, and allowed to react at 100 °C for 15 minutes. Finally, samples were placed in cuvette, analyzed using a plate reader at 525 nm absorbance wavelength, and compared to standard curves to determine degradation plots.

Fluorescence Recovery After Photobleaching

Fluorescence recovery after photobleaching (FRAP) experiments were conducted on a confocal microscope (TCS SP5, Leica, Wetzlar, Germany). In each experiment, 20 μL hydrogels were placed on glass slides and covered with a glass cover slip. The 488 nm line of an argon laser was set to 50 % power and all images were taken using the 10× objective with the pinhole fully opened. Pre-bleach images were recorded over 2 seconds using 0.1 % transmission. A 30 μm diameter region was then bleached for 4 seconds using 100 % transmission. Post-bleach images were then recorded with the laser returned to 0.1 % transmission for 230 s of recovery. Data was analyzed using a custom MATLAB script that fit recovery profiles using nonlinear least squares regression to the Soumpasis equation:

$$F(t) = k \bullet e^{-\frac{\tau_D}{2t}} \left[I_0\left(\frac{\tau_D}{2t}\right) + I_1\left(\frac{\tau_D}{2t}\right) \right] \quad (1)$$

where $F(t)$ is the normalized fluorescence recovery profile, k is the mobile fraction, τ_D represents the characteristic diffusion time (s), t represents time (s), and I_0 and I_1 are zero and first order modified Bessel functions of the first kind.[30] Effective diffusivities were then calculated according to:

$$D_{eff} = \frac{w^2}{\tau_D} \quad (2)$$

where D_{eff} is the effective diffusivity ($\mu m^2\ s^{-1}$) and represents the bleach spot radius (μm). This protocol was adapted from previous work conducting similar analyses.[31]

Molecule Release Profiles

Release assays were conducted in custom made acrylic erosion cells. Erosion cells were fabricated with a 4.3 mm diameter hydrogel chamber overlaid with a 1.6 cm diameter supernatant chamber, each chamber having depths of 7 mm and 10 mm, respectively. 30 μL of the hydrogel was deposited in the chamber, the chamber was centrifuged to provide an even hydrogel surface, and the hydrogel was covered with 1 mL of PBS. Once loaded, erosion cells were stored in an incubator at 37 °C. Supernatant was collected at regular intervals and replaced with fresh PBS. For the covalently crosslinked MeHA gels, a similar approach was used where 30 μl of MeHA containing 100 μM 3W peptide was polymerized into the erosion cell and supernatant collected as above. The final time point was obtained by degrading the remaining MeHA hydrogel in 1 mg/mL hyaluronidase solution.

Molecule release was determined with a plate reader (Infinite M200, Tecan, Männedorf, Switzerland). FITC labeled peptides were analyzed using 495/525 nm excitation emission, doxycycline was analyzed using 350 nm absorbance, and doxorubicin was analyzed using 480/590 nm excitation/emission. At the end of each assay, any remaining hydrogel was collected along with supernatant and disassociated with addition of 5 mg/mL Ad solution in DMSO to obtain final fluorescence/absorbance readings. Fluorescence and absorbance values were then compared to standard curves to determine total molecule release.

Statistical Analysis

Affinity measurements are presented as an average of affinity constants determined from n = 3 isotherms per trial. Mechanical measurements are presented as an average and standard deviation for n = 3 samples. FRAP measurements were conducted for n = 4 recovery profiles, and presented as average effective diffusivities and standard deviations. Release profiles are presented as average cumulative percent release and standard deviation of cumulative release for n = 4 samples. Statistical analyses on ITC and rheology measurements were conducted using two-tailed student t tests. One-way ANOVA with post-hoc Tukey's Honestly Significant Difference testing was conducted on diffusivity measurements using R.

Results and Discussion

Small molecule binding to HA

HA was successfully functionalized with CD to produce CD-HA, with ~35 % of HA repeat units modified. It was first investigated whether CD modification of HA could increase the polymer affinity for small molecules, using ITC. As a representative small molecule, L-tryptophan was used to investigate this interaction, due to its aromatic structure, hydrophobic nature, and previous investigation of affinity with CD.[15, 24, 26] Additionally, as a natural amino acid, L-tryptophan presents an interesting binding target, as it allows for peptide design with natural compounds that may promote polymer affinity.

Isotherms produced by CD-HA and unmodified HA titrations were vastly different (Figure 2A and 2B). CD-HA produced a distinct exothermic reaction upon mixing with tryptophan in the sample cell, while unmodified HA remained at near baseline levels for all injections. Isotherms were quantitatively analyzed in Origin 7 software by a one-binding site model fit to determine the apparent association constant (K_A) between the polymer and tryptophan. The binding site stoichiometry value (n) was set to 0.35 to account for CD modification as determined by ^{1}H-NMR and to reduce error associated with fitting multiple parameters. Fits for isotherms of CD-HA and tryptophan produced an average K_A of $1.6\times10^4 \pm 2.9\times10^3$ M^{-1}. While this value is slightly higher than previously reported values for this interaction, the increase may be attributed to changes in CD chemistry upon conjugation to HA, as it has been shown that these changes can alter binding affinities to tryptophan.[26] Accurate values for K_A were not attainable in unmodified HA isotherms due to high error associated with parameters obtained from fits, which is attributed to randomly scattered integrated heat values resulting from near baseline heat changes for all injections. Taken together, these data suggest that modification of HA with CD provides a multiple orders of magnitude increase in affinity for tryptophan. Since the cyclic domains such as the indole moiety found in tryptophan are found in many pharmacologic small molecules, this increase in affinity suggests that HA modification would allow increased affinity for many small molecules.[32]

Effect of drug loading on hydrogel mechanics

HA was also successfully modified with Ad groups to form Ad-HA. Gelation occurred instantaneously upon mixing of CD-HA and Ad-HA macromer solutions, through host-guest interactions of Ad and CD groups. Since incorporated small molecules also complex with CD, it is important to understand the competitive effect of drug loading on hydrogel mechanics. As a model drug, a peptide modified with tryptophan and FITC (termed 3W, due to 3 tryptophan residues per peptide) was loaded (1 mg/mL) into 1:1 (5 wt%) hydrogels. This notation of the form ‘x:y (z wt%)’ refers to the molar ratio of Ad:CD in the hydrogel (x:y) and the total polymer weight percent in the hydrogel (z). This formulation was selected for mechanical testing since it would have the greatest chance to reduce hydrogel mechanics with drug loading, as the Ad:CD ratio is 1:1. The loading concentration for 3W of 1 mg/mL is at the peptide solubility limit and is ~5 % of the total CD content in the hydrogel. Furthermore, this concentration is orders of magnitude higher than loading concentrations used in subsequent release assays. Please note that the peptide was not designed for any

bioactivity, but rather for use as a model molecule to investigate the influence of hydrogel design on diffusivity and release.

To examine the effect of molecule incorporation on hydrogel mechanical properties, frequency response profiles for G', G", and tan(δ) were obtained. Hydrogels displayed frequency responsive behaviors that are characteristic of self-assembling, two-component systems (Figure 3A). Both molecule-loaded and unloaded hydrogels exhibited similar frequency response profiles for these parameters, showing qualitative similarity in mechanics. Time sweeps were conducted to assess differences between these two groups in shear and loss moduli at 0.1 Hz, 1 Hz, and 10 Hz. Both formulations displayed frequency responsive increases in storage moduli (Figure 3B) and loss moduli (Figure 3C) and there were no significant differences in either storage or loss moduli ($p > 0.05$) between the formulations. One explanation for the lack of difference in bulk mechanics with and without encapsulated small molecules is the relative difference in affinity of tryptophan for CD as compared to AD. The association constant for the Ad-CD complex has been reported to be as high as 10^5 M^{-1}, orders of magnitude higher than previous reports for tryptophan-CD, suggesting that there would be minimal competition between the two.[15, 26] Considering that a wide variety of small molecules complex with CD on the same order of magnitude as tryptophan, it is unlikely that loading of therapeutics would compete with the formation of the Ad-CD complex and cause a decrease in hydrogel mechanics.[15]

Finally, loaded hydrogels were subjected to a cyclic strain protocol, whereby hydrogels were placed under cycles of high and low oscillatory strain to simulate conditions during injection and recovery after injection. This technique has been used previously to examine network disassembly and reassembly in self-assembling systems from a mechanical perspective.[33-36] This analysis is representative of the disassociation between the guest and host pair on the polymer chains with high strain (such as at the walls of a delivery device, e.g., syringe) and the corresponding self-healing that occurs when the strain is decreased (once the gel reaches the target location).

Hydrogels showed a rapid decrease in storage modulus under high strain, indicating a reduction in elastic properties and a more fluid-like behavior, as well as a rapid recovery of the storage modulus under low strain, suggesting a return to a more elastic, gel-like state (Figure 3D). Hydrogels loaded with the peptide rapidly assembled after injection through a 27G needle (Figure S2), indicating that there would be minimal extravasation during administration. These characteristics are important in the development of translational therapies, so that both hydrogel and drugs may be precisely localized. This aspect can be challenging with alternative injectable hydrogels that covalently crosslink at the injection site, as hydrogels may clog the delivery device if gelation occurs too quickly or may diffuse from the injection site prior to gelation if gelation is too slow.[6]

Effect of molecule and hydrogel parameters on drug mobility

Fluorescence recovery after photobleaching experiments were used to assess encapsulated molecule mobility within hydrogels as a function of hydrogel parameters and molecule interaction with CD. Using this technique, small regions of hydrogels loaded with mobile fluorescent payloads were bleached with a high intensity laser. The recovery of fluorescence

due to diffusion of unbleached molecules into the region was then monitored and quantified to determine the diffusivity of the fluorescent payload within the hydrogel. Two fluorescently labeled peptides were used in these experiments: a high affinity peptide for CD (3W), as well as a lower affinity peptide, in which the tryptophan residues were replaced with glycine (NoW, GKGEGKGE-FITC). These peptides were used as model drugs to represent payloads of varying molecular affinities, and were loaded into hydrogels at 100 μM.

To assess the influence of hydrogel formulation on molecule mobility, CD content within hydrogels was altered by increasing the overall CD-HA concentration (1:1 (5 wt%), 1:2 (7.8 wt%), and 1:3 (10.5 wt%)). As CD concentration increased, the effective diffusivity of the 3W peptide significantly decreased within the hydrogel (Figure 4A). These data suggest that the increase in CD-HA content does reduce peptide mobility within the hydrogel. To address the potential confounding effects that an increase in total macromer concentration may have on mobility, an additional formulation at a 1:2 Ad:CD ratio was compared to the 1:1 Ad:CD ratio, with both hydrogels at 5 wt%. The 1:2 Ad:CD formulation showed significantly lower effective diffusivities than the 1:1, despite maintaining a similar total macromer concentration of 5 wt% (Figure 4B). This comparison suggests that the CD-HA component itself is a significant contributor to molecule diffusivity, which is supported by the binding affinity results quantified by ITC analysis.

To further investigate this affinity mediated reduction in mobility, we explored the effect of removing the tryptophan residue from the 3W peptide by using the NoW peptide. A 1:2 (7.8 wt%) hydrogel was loaded with 100 μM NoW peptide and compared to a similarly formulated hydrogel with 100 μM 3W peptide. It was observed that the NoW peptide had significantly higher diffusivity in the same hydrogel when compared to the 3W for the given 100 μM concentration (Figure 4C). These results can be observed visually in Figure 4D. Both peptides were bleached with similar efficiencies (1 s), but show differences in recovery profiles (3 s and 8 s) until both reach almost full recovery (30 s). The influence of peptide chemistry on mobility further implies the importance of CD affinity in controlling peptide mobility in these hydrogels.

Effect of molecule and hydrogel parameters on bulk release profiles

Towards investigating the small molecule sustained release properties of these hydrogels, we next investigated the effect that both hydrogel and payload parameters have on bulk release profiles. As in the FRAP experiments, groups were selected to compare the effects of increasing CD-HA concentration, increasing the Ad:CD ratio for a given total weight percent, and changing the relative affinity of a fluorescently labeled payload. In order to provide a contrast to this affinity-based interaction, release from a covalently crosslinked hydrogel with no CD content was examined. MeHA hydrogels containing no CD content were formed through the photoinitated polymerization of 30 % modified MeHA at 7.8 wt% and containing 100 μM of the 3W peptide. Peptide release into PBS was examined over 8 days. This profile (Figure S3) showed ~90 % release in the first 24 h and exemplifies the issues with sustaining small molecule release from traditional swollen hydrogels.

Towards investigating the cyclodextrin retentive effect in our hydrogels, several formulations were explored. All supramolecular hydrogels discussed here were loaded with 100 μM of peptide. This represents approximately a 1:100 ratio of peptide to CD available for binding (i.e., CD that has not been paired with Ad) in a 1:2 (7.8 wt%) system. This ratio is consistent with values investigated in previous modeling studies investigating affinity mechanisms in drug release.[37] The effect of increasing CD-HA macromer concentration was assessed in these hydrogels by conducting release experiments on a 1:1 (5 wt%), 1:2 (7.8 wt %), and 1:3 (10.5 wt%) hydrogels. Results of these systems are shown in Figure 5A. Analogous to trends observed in molecule diffusivity from FRAP studies, increasing the CD-HA content of these hydrogels produced slower bulk release kinetics. In the first 24 h, ~70 %, ~35 % and ~20 % of the initial payload was released for the 1:1 Ad:CD, 1:2 Ad:CD, and 1:3 Ad:CD systems, respectively. These results are markedly improved over the MeHA system, and show the increased ability of mismatched systems to sustain the release of small molecule payloads. Furthermore, even matched systems, where there is an equal molar ratio of Ad and CD, present some sustained release capabilities. This is likely due to spatial limitations in the network that prohibit complete consumption of CD by Ad guests, leaving the remaining CD available to bind to drugs. It should also be noted that only marginal improvements in sustained release were observed when shifting from 1:2 to 1:3 Ad:CD hydrogels. Likely this is due to an upper limit on retentive effects that can be achieved through CD-peptide host-guest interactions for this particular payload and concentration. These materials also displayed moderate erosion (due to disassociation of polymer chains from the hydrogel) during the three-week assay, and are comparable to our previous reports on the degradation of this material (Figure S4). [3]

A comparison of release profiles between a 1:1 (5 wt%) to a 1:2 (5 wt%) hydrogel (Figure 5B) further indicates that the sustained properties are a result of CD content within the hydrogel. A mismatched system of the same macromer concentration produced slower sustained release profiles, which can be attributed to the increased amount of unbound CD available to bind and retain the peptide. Finally, the effect of varying peptide affinity on bulk release was investigated. Hydrogels were formulated at 1:2 (7.8 wt%) and 100 μM concentration of either 3W or NoW peptide. Release over 21 days was markedly slower for 3W when compared to the NoW peptide (Figure 5C). However, the NoW peptide still shows some degree of sustained release from these hydrogels, since CD binds with a number of small hydrophobic moieties such as the fluorescein dye on NoW. Release data of these peptides with varying structure further reinforces the importance of CD affinity in these systems.

Overall, these release studies have elucidated a number of properties about these hydrogels and the release of small molecules. First, our self-assembled hydrogels provided sustained release of two different peptides for up to 3 weeks. This shows remarkable improvement over the capacity of traditional systems (i.e., hydrogels without a drug affinity component) to sustain the release of molecules of this size (Figure S3). These systems have tunable profiles that can be changed through a number of parameters. Engineering molecule affinity is one mechanism that can provide specific control of release profiles on a drug-to-drug basis. This is useful particularly in the delivery of peptide therapeutics, as these can be readily engineered with groups such as tryptophan that will promote the appropriate release

profile. Finally, and most importantly, by varying the amount of CD-HA macromer in these hydrogels, the extent of molecule retention and subsequent release can be easily controlled. These multiple controllable parameters allow for potential tunability over a wide range of molecules as payloads.

Sustained release of doxycycline and doxorubicin

To show the application of these systems towards small molecule pharmacologics, two clinically used small molecule therapeutics (i.e., DOX, DXY) were investigated. These drugs were selected as DOX is a chemotherapeutic that acts through intercalating with DNA and DXY has been investigated as an MMP inhibitor, both classes of drugs that benefit from localized delivery and both have been shown previously to form complexes with CDs and their derivatives.[22, 25, 38-40] Illustration with these varied drugs was intentional to show diversity in the potential application of such an injectable therapeutic delivery vehicle, which could have widespread use for the wide range of small molecules that have been previously developed.

Both the 1:1 (5 wt%) and a 1:2 (7.8 wt%) hydrogel formulations were investigated for release, with drug loading at 1 mg/mL. DOX release was sustained over the first 14 days of the study (Figure 6A). As observed with fluorescently labeled peptide substrates, increasing CD-HA content in DOX loaded hydrogels produced a similar reduction in release kinetics, particularly noticeable during the first week. DXY loaded hydrogels also showed similar trends to the DOX loaded systems (Figure 6B). The sustained release effect of increased CD-HA content was more pronounced in DXY release profiles, and this may be attributed to differences in CD affinity between DXY and DOX, which have been reported at 503 M^{-1} and 297 M^{-1}, respectively.[22, 41] Profiles for the 1:2 Ad:CD formulation for both drugs also indicate incomplete release of the encapsulated payload, with an increased fraction of drug retained in the hydrogel at the study endpoints. These data show the versatility of the presented hydrogel system in delivering a number of small molecules in a readily translatable manner. As injectable systems, hydrogels providing sustained release of either of these two molecules could find biomedical applications, such as in cancer therapies.[39, 42] Although the work here did not illustrate bioactivity of released drugs, we do not expect any comprised activity since we have not chemically modified the drugs and others have shown activity of molecules in delivery systems leveraging non-covalent inclusion complexes.[43, 44]

Conclusion

Here, we have developed an injectable hydrogel system for the tunable and sustained release of small molecules using CD host-guest interactions. To our knowledge, no other material system has combined the benefits of an injectable hydrogel with sustained small molecule release through inclusion complex formation between the drug and hydrogel. Modification of HA with CD increased affinity for model small molecules by multiple orders of magnitude, and this affinity was leveraged to tune the release of peptide model drugs, as well as doxorubicin and doxycycline, from assembled hydrogels. Furthermore, hydrogel drug-loading had minimal effects on mechanics and allowed the hydrogels to retain their injectable and rapid self-assembly properties. Ultimately, the work presented in this paper

shows a small molecule, controlled release system that is rapidly formulated, easily tuned, and avoids the potential challenges associated with other injectable delivery systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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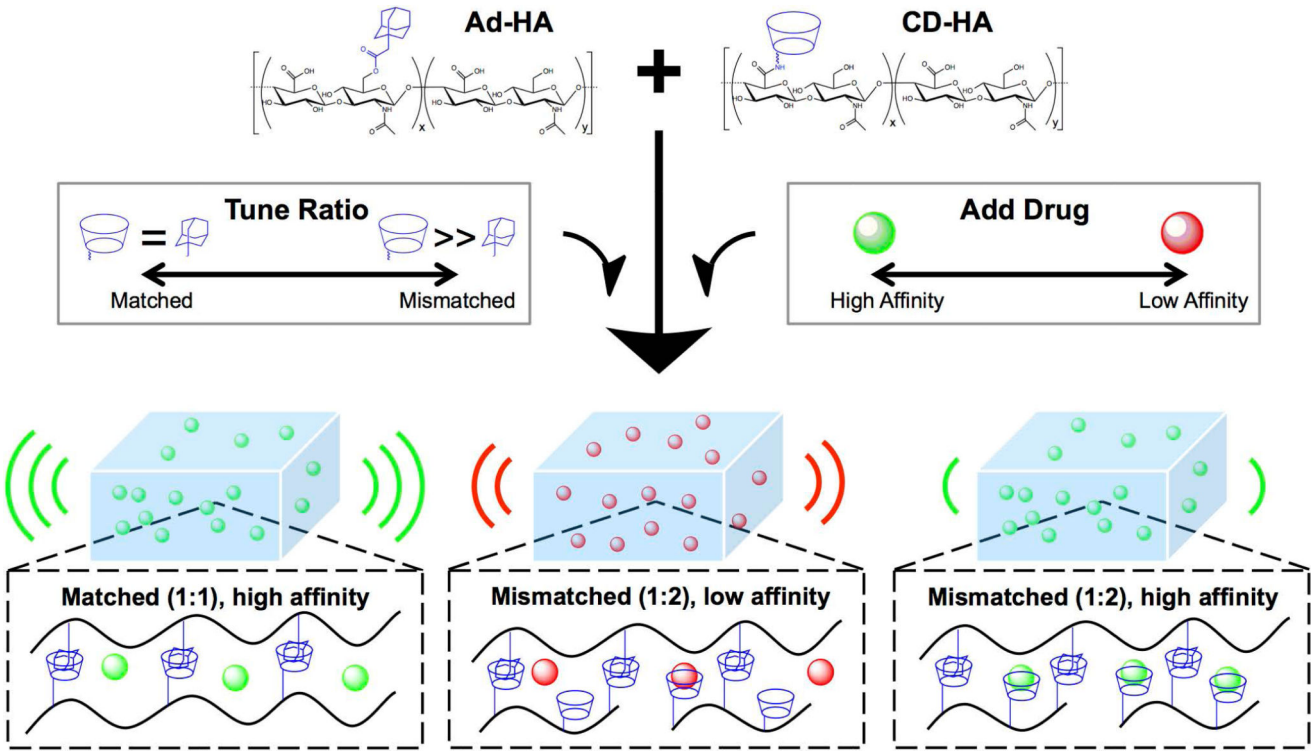

Figure 1.

Scheme for tuning release of small molecules from drug loaded supramolecular hydrogels. Ad-HA and CD-HA macromers were synthesized and evaluated for functionalization (x/[x +y]) using ^{1}H-NMR. Macromers were dissolved in solutions containing varying drugs (green = high affinity for CD, red = low affinity for CD) and combined in varying ratios (Ad:CD) to produce bulk hydrogels. Release kinetics were tuned by varying the CD content available for drug binding or by altering the affinity of the loaded drug for CD.

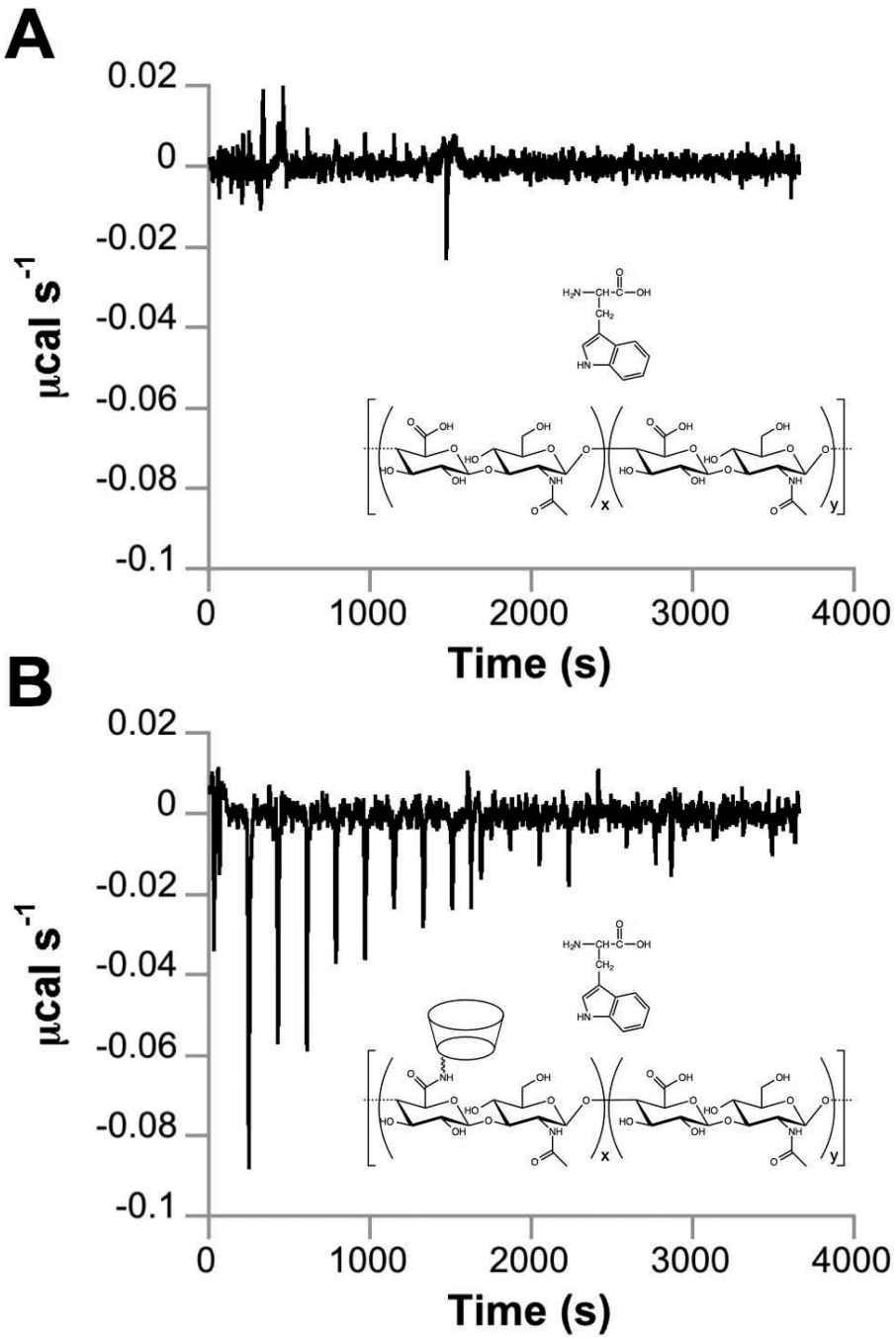

Figure 2.

Isothermal titration calorimetry for HA binding to tryptophan. Solutions of HA (either unmodified or modified with CD) dissolved in MilliQ water were titrated into 40 μM tryptophan in MilliQ water. Baseline isotherms of modified or unmodified HA into water were subtracted to account for heats of dissolution. Representative isotherms and chemical structures of binding components are shown for (A) unmodified HA binding to tryptophan or (B) CD-HA binding to tryptophan.

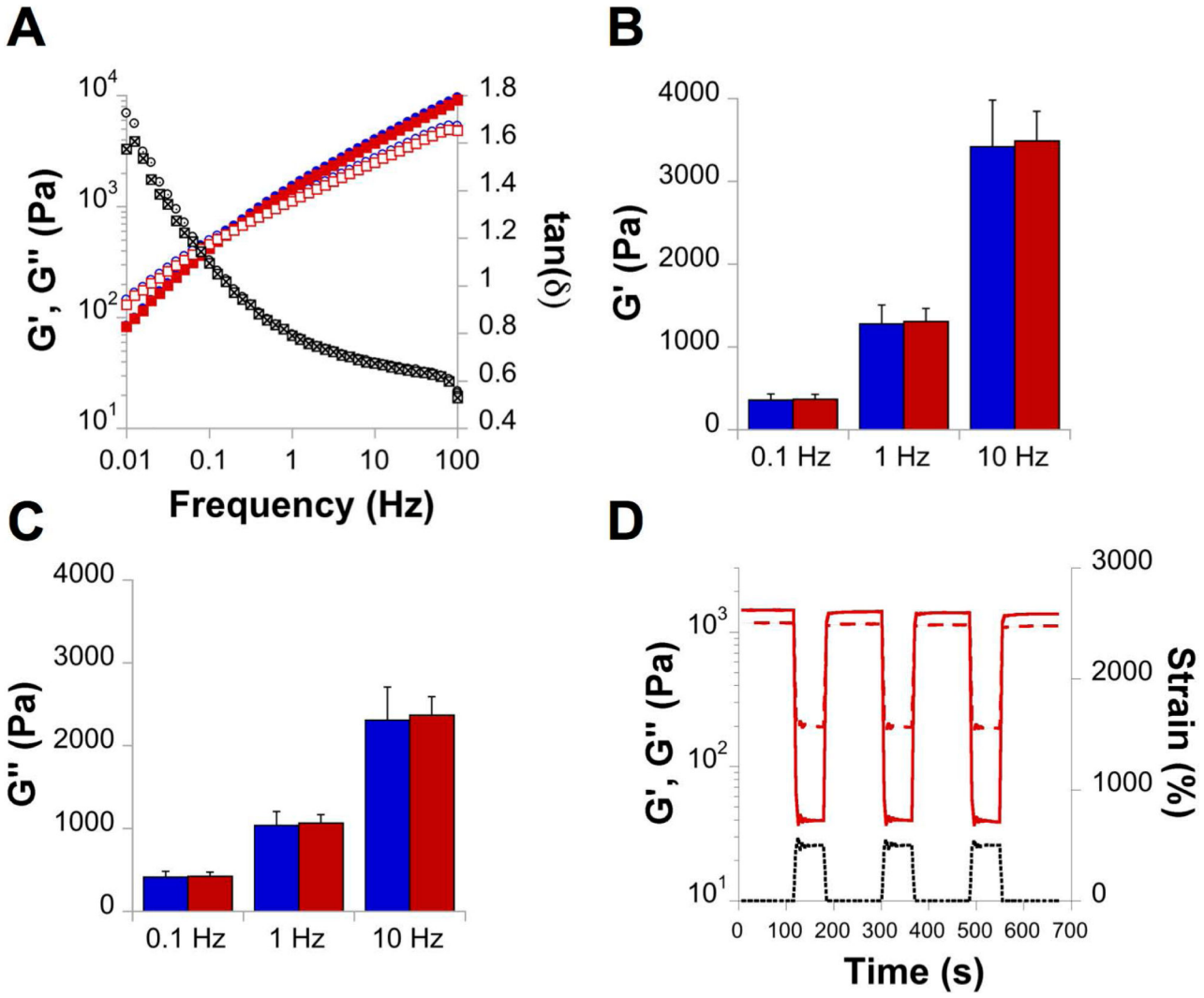

Figure 3.

Comparison of mechanical properties in unloaded and drug loaded (3W peptide, 1mg/mL) hydrogels (1:1 (5 wt%)). (A) Representative frequency sweeps showing G' (unloaded=blue solid circles, loaded=red solid squares), G" (unloaded=blue open circles, loaded=red open squares), and tan(δ) (unloaded=black open circles, loaded=black crossed squares). (B) Average G' values for time sweeps for unloaded (blue) and loaded (red) hydrogels for 0.1Hz, 1Hz, and 10Hz frequencies. (C) Representative cyclic strain experiment showing G' (red solid line), G" (red dashed line), and strain (black dotted line) for loaded hydrogels undergoing cycles of low and high strains.

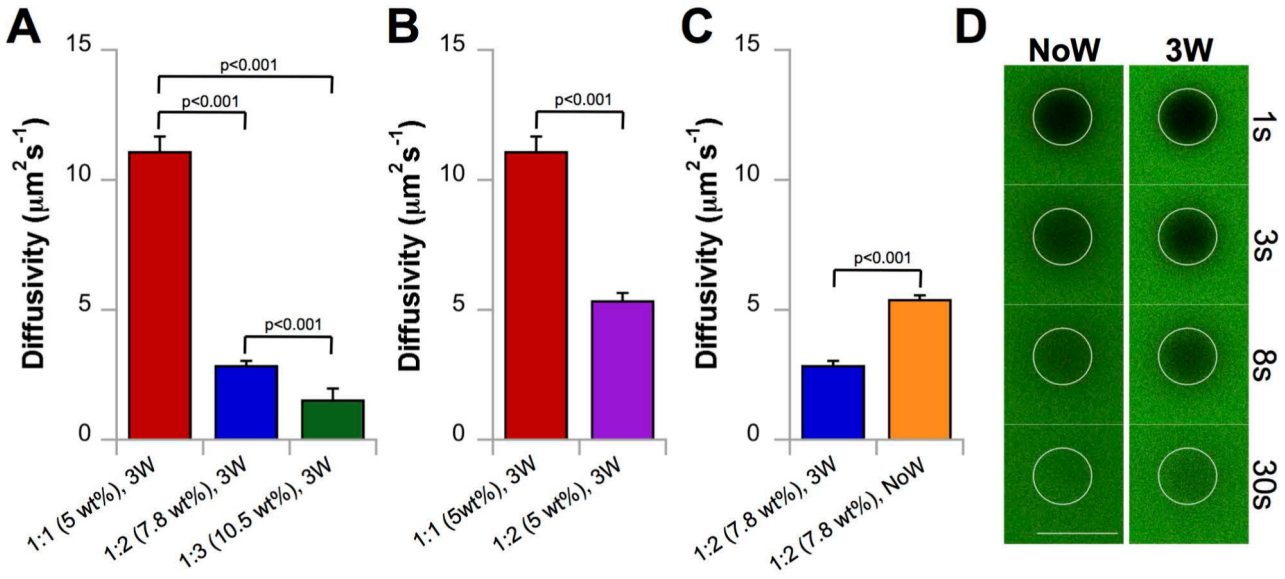

Figure 4.

Fluorescence recovery after photobleaching experiments. Hydrogels loaded with fluorescent peptides were assessed with FRAP analysis to determine payload mobility within the hydrogel. Diffusivity of fluorescent peptide payloads in hydrogels was assessed with changes in (A) CD-HA wt%, (B) Ad:CD ratio at a total polymer concentration of 5 wt%, and (C) payload chemistry (3W versus NoW). (D) Representative FRAP images for changes in payload chemistry, scale bar = 50 μm.

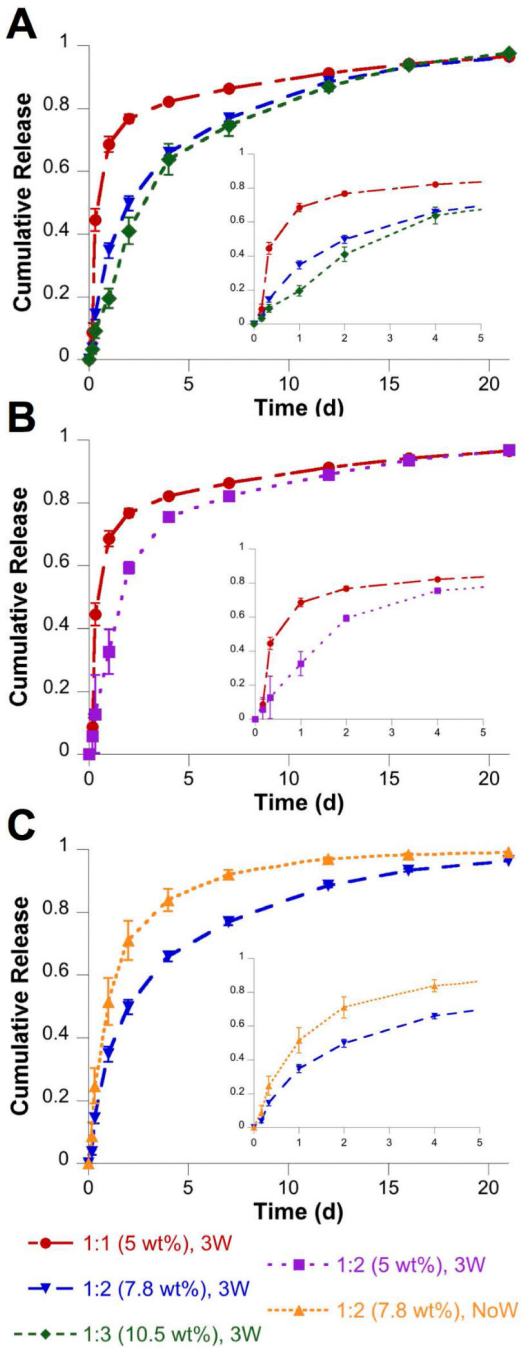

Figure 5.

Release profiles of entrapped peptides. Hydrogels containing fluorescent peptides were injected in custom designed erosion cells and peptides released into PBS were quantified. Cumulative release profiles were determined in gels with changes in (A) CD-HA wt%, (B) Ad:CD ratio at total polymer concentration of 5 wt%, and (C) payload chemistry (3W versus NoW). Insets show an expanded view of time points collected during the first 5 days.

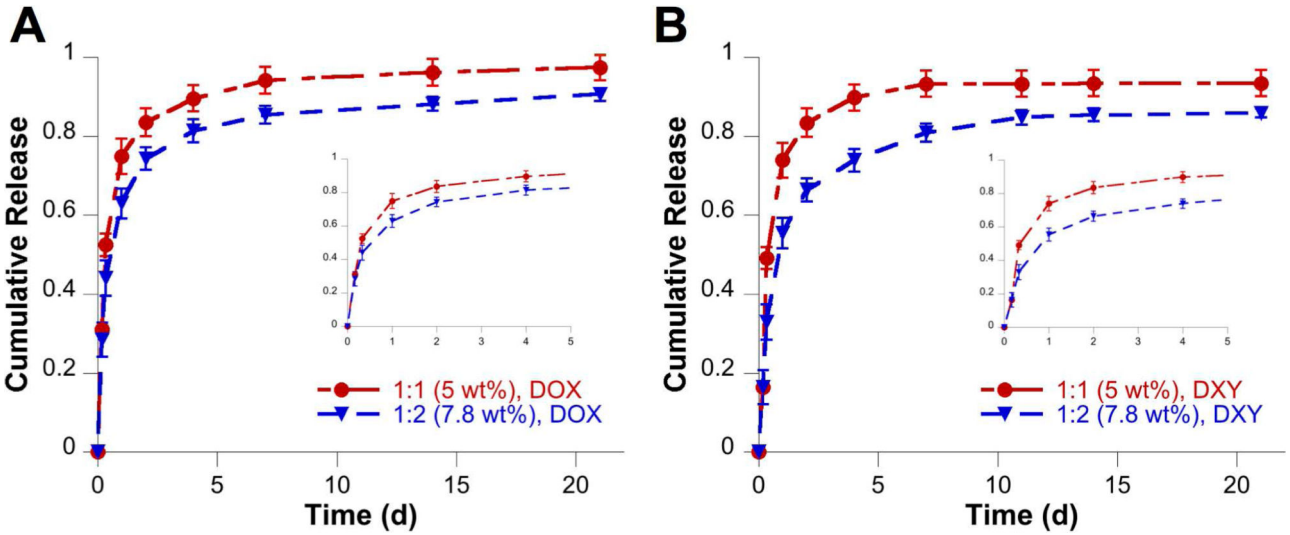

Figure 6.

Release of doxorubicin (DOX) and doxycycline (DXY) from injectable hydrogels. Cumulative release of (A) DOX or (B) DXY for 1:1 (5 wt%) (red) or 1:2 (7.8 wt%) (blue) formulations over 3 weeks. Insets show an expanded view of time points collected during the first 5 days.