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Supplementary Information for:

Enhanced User-Control of Small Molecule Drug Release from a Poly(ethylene glycol) Hydrogel via Azobenzene/Cyclodextrin Complex Tethers

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1. Materials and Methods

All reagents were purchased from Sigma-Aldrich and used without further purification unless otherwise noted.

A. Synthesis of 3-[[4-([[(9H-Fluoren-9-ylmethoxy)carbonyl]-amino]methyl)pen-yl]diazenyl]benzoic Acid (3,4'-Fmoc-AMPB)

3,4'-Fmoc-AMPB was synthesized in two steps as previously published and shown in Scheme S1[1–3]. In the first step, 3-nitrosobenzoic acid was synthesized by dissolving 2 g of 3-aminobenzoic acid in 40 mL of dichloromethane (DCM). Next, 2 equivalents of Oxone were dissolved in 160 mL of water and added to the reaction flask, resulting in a biphasic solution. The solution was allowed to react in an agitated flask for 3 hours at room temperature with a continuous argon purge. A precipitate formed and, after vacuum removal of the DCM, was centrifuged and washed two additional times with cold water and two times with methanol. The solid pellet was then dried overnight in a vacuum oven at 40 °C. The ^{1}H NMR spectrum of this compound is shown in Fig. S1. ^{1}H NMR, DMSO-d_6, 500 MHz: δ = 7.88 (m, 1H), 8.19 (m, 2H), 13.52 (s, br., 1H)

In the following step, 2 g of the 3-nitrosobenzoic acid was dissolved in 100 mL of a 1:1 mixture of dimethyl sulfoxide (DMSO) and acetic acid. To the resulting green solution, 0.5 equivalents of 4-(N-Fmoc-aminomethyl)aniline was added, and the condensation was stirred under argon for three days at room temperature. The resulting precipitate was centrifuged into a solid pellet, dissolved in ethyl acetate, and washed with water. The organics were dried over $MgSO_4$ and the solvent was evaporated. The resulting product was used without further purification. Final ^{1}H NMR results of this synthesis are shown in Fig. S2. ^{1}H NMR, DMSO-d_6, 500 MHz: δ = 4.26 (t, 1H), 4.29 (d, 2H), 4.40 (d, 2H), 7.35 (t, 2H), 7.43 (m, 4H), 7.73 (m, 3H), 7.90 (d, 2H), 7.91 (d, 2H), 7.99 (t, 1H), 6.14 (m, 2H), 8.39 (s, 1H)

B. Synthesis of Guest Peptide

The desired azobenzene functionalized guest peptide, shown in Fig. S3a, was synthesized in 0.25 mmol batches on a solid phase peptide synthesizer (Tribute Protein Synthesizer, Protein Technologies, Inc) on Rink Amide MBHA resin (Novabiochem, 0.59 mmol/g). Fmoc-protected amino acid residues, E and Q, were added in 4 equivalents to resin binding sites (CHEM-IMPEX). [2-[2-(Fmoc-amino)-ethoxy]-ethoxy]acetic acid (Fmoc-AEEAc-OH, AnaSpec) residues were used as PEG spacers and were added in 3 equivalents. 3,4'-Fmoc-AMPB was added in 2 equivalents. Fmoc deprotection was carried out for 20 minutes in 20% piperidine/2% 1,8-diazabicyclo[5,4,0]-undec-7-ene in N-methylpyrrolidone (NMP) (CHEM-IMPEX). Residue coupling was carried out for 30 minutes in 0.4 M N-methylmorpholine in dimethylformamide (DMF) (Applied Biosciences). An acetylation step was performed using 5% acetic anhydride and 6% lutidine in NMP after the addition of each intermediate residue and after addition of Fmoc-AMPB. The tether was cleaved from the resin in a 95% TFA cocktail that contained 2.5% phenol, 1.25% triisopropylsilane, and 1.25% water for 2 hours on an orbital shaker. The result was precipitated into cold diethyl ether, centrifuged for 5 minutes at max speed, dissolved in 20% acetonitrile (ACN) in H_2O, and purified by semi-preparative reverse-phase high performance liquid chromatography (AutoPurification HPLC, Waters) using a 10 minute gradient of acetonitrile in water (5-50%, 30 mL/min). Mass was verified by matrix assisted laser desorption ionization time-of-flight spectrometry (MALDI-TOF, (exp/obs (m/z): 843.33/843.56) with a α-cyano-4-hydroxycinnamic acid (α-CHCA) matrix (Fig. S3b). The HPLC fractions that contained the desired tether were combined and lyophilized in preparation for later use.

C. Determination of Association Constant, K_a

K_a is generally given by Eq. S1, where [G] refers to the concentration of guest at equilibrium, [H] refers to the concentration of host at equilibrium, and [H·G] refers to the concentration of the complex at equilibrium. Since these values are not directly measurable, spectroscopic methods have been developed to calculate this value. An NMR adapted Benesi-Hildebrand method was used to determine K_a for the association of Guest and β-CD[4,5]. To achieve this, samples were prepared in deuterium oxide that contained 0.5 mM guest tether and between 0 and 20 molar equivalents of β-CD. The results were then plotted according to Eq. S2, where $\Delta\delta = \delta_G - \delta_{Observed}$ and $\Delta\delta_{max} = \delta_G - \delta_{H\cdot G}$. From this plot, $\Delta\delta_{max}$ can be calculated from the intercept and used in the computation of K_a from the slope. The shift was measured from the protons of the azobenzene guest as seen in appendix Fig. S2.

$$K_a = \frac{[H \cdot G]}{[H][G]} \quad \text{S1}$$

$$\frac{1}{\Delta\delta} = \frac{1}{K_a \Delta\delta_{max}[H]_0} + \frac{1}{\Delta\delta_{max}} \quad \text{S2}$$

D. Demonstration of Reversibility

To demonstrate the reversible nature of the inclusion complex formation, an ^{1}H NMR technique was employed, capitalizing on the same peak shifts that lead to the adaptation of Benesi-Hildebrand to NMR. In this study, an NMR sample was prepared in D_2O with a Guest concentration of 0.5 mM and 20 molar equivalents of β-CD. Samples containing only 0.5 mM guest or 10 mM β-CD were also prepared and underwent the same treatment for comparison. Initial spectra were taken prior to any irradiation. The samples were irradiated with an OmniCure UV/Vis spot curing lamp at an intensity of 5 mW/cm^2 and wavelength of 365 nm for 5 minutes, and spectra of the irradiated samples were taken. Finally, the samples were irradiated again with the same intensity and duration but using a wavelength filter of 400-500 nm, and spectra were taken. The resulting plot can be found in Fig. 3b. Since the NMR results from the negative control did not change upon irradiation and contained no peaks that would interfere with calculations in the region of interest, the corresponding spectra were not included. From this plot, Δδ was calculated for each condition.

E. Preparation of PEG-CD

3A-Amino-3A-deoxy-(2AS,3AS)-β-cyclodextrin Hydrate, denoted β-CD, TCI) and was reacted with Traut's reagent (2-iminothiolane) for 1 hour at room temperature in PBS (pH 8). The reaction was stopped by HPLC purification in a ten minute, 5-50% ACN/H_2O gradient (30 mL/min). 8-arm 10 kDa PEG-acrylamide (Creative PEGWorks) was reacted overnight at room temperature with the newly thiolated cyclodextrin in PBS (pH 10) with 0.3 M triethanolamine (TEOA). The resulting solution was purified by dialysis in 2L of dH_2O in a 3000-5000 Da cellulose membrane (Spectrum Laboratories) for two days, changing the water after 2 and 24 hrs. The purified solution was lyophilized to obtain the resulting PEG-CD as a powder. Degree of functionalization was determined to be 1.6 CD host molecules per 8-arm PEG star by ^{1}H NMR (500 MHz) in DMSO-d_6 (Fig. S4).

F. Fluorescence Labeling of Guest

Fluorescence labeling took place by adding 1 mL of 1.18 mM guest peptide dissolved in anhydrous dimethylformamide (DMF) to an evacuated and argon purged flask, and subsequently adding 0.67 molar equivalents of N-hydroxysuccinimide activated Alexa Fluor 647 (Invitrogen) dissolved in 0.5 mL of

anhydrous DMF and 50 uL of diisopropylethylamine (DIEA). The reaction mixture was stirred for 48 h, protected from light. The solution was diluted to 20 mL with 20% ACN in HPLC grade H_2O and purified by HPLC on a 5-95% ACN gradient over 17 minutes. The desired peptide was collected in 40-50% ACN and its identity was verified by a UV-Vis spectrum that contained the signature azobenzene peaks at 325 nm and 450 nm, as well as a very strong peak at 647 nm corresponding to the fluorophore. A powder was obtained via lyophilization and stored at -20 °C.

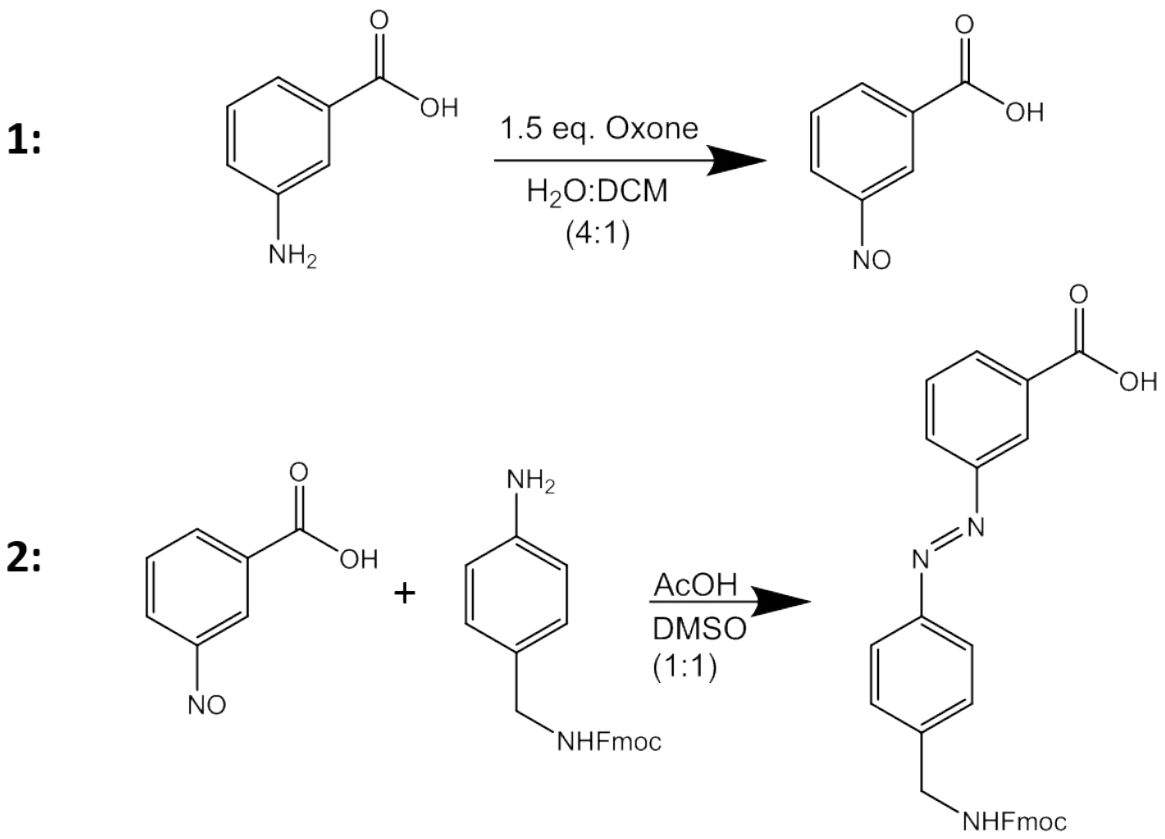
G. Hydrogel Formation

Robust hydrogels were formed via Michael-type reaction of PEG-CD with 8-arm, 10 kDa PEG-thiol (JenKem Technology). The reagents were dissolved separately at 10% w/v concentration in PBS (pH 10) with 0.3 M TEOA, mixed together in a 1:1 ratio of thiol to acrylamide, and vortexed to mix in a 0.65 mL microcentrifuge tube. The result was injected into a 0.5 mm rubber gasket (8mm diameter) between two SigmaCoated glass slides and allowed to react for 3 hours at 37 °C.

H. Release Studies

To generate profiles for the release of the labeled Guest from a 10 wt% CD containing gel, 20 µL 14 mg/mL labeled Guest was mixed with 40 µL of a 15 wt% gel solution prepared as described above from PEG-CD and PEG-thiol. This gel was allowed to form for 3 hours at 37 °C, protected from light. The gels were then transferred into 0.5 mL PBS (pH 7). The gels were left in on a lab bench during the release studies, and 2 µL samples were taken at the designated time points for analysis on a NanoDrop (ND-1000) spectrophotometer. For gels that were subjected to pulsed irradiation, 365 nm light (5 mW/cm^2) from the OmniCure was continuously directed at the gel for the first 5 minutes of every 15 minute interval. For analysis, it was assumed that equilibrium had been reached after 24 hours, and the fraction released was calculated by normalizing to the fluorescence reading at 647 nm after 24 h in solution. All error bars represent one standard deviation from the mean.

2. Synthetic Scheme for 3,4'-AMPB[1]



Scheme S1: Two step synthesis of 3,4'-AMPB.

3. ^{1}H NMR of 3-Nitrosobenzoic Acid

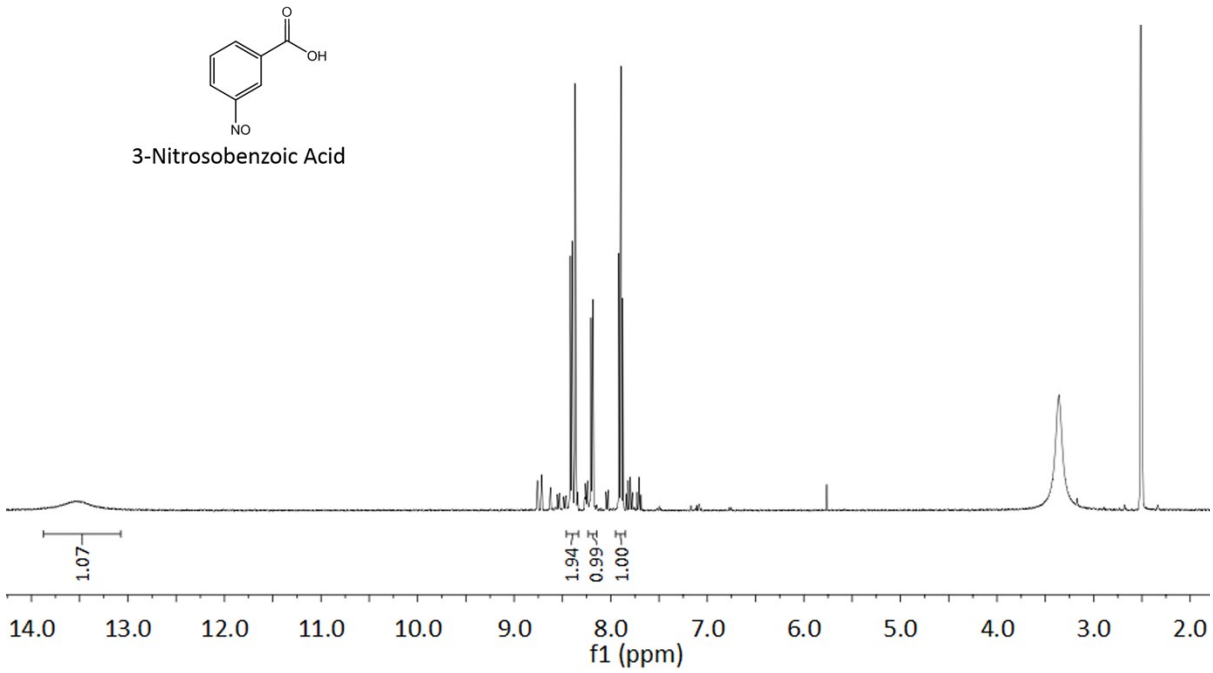


Fig. S1: ^{1}H NMR of 3-Nitrosobenzoic Acid in DMSO-d_6.

4. ^{1}H NMR of 3,4’-AMPB

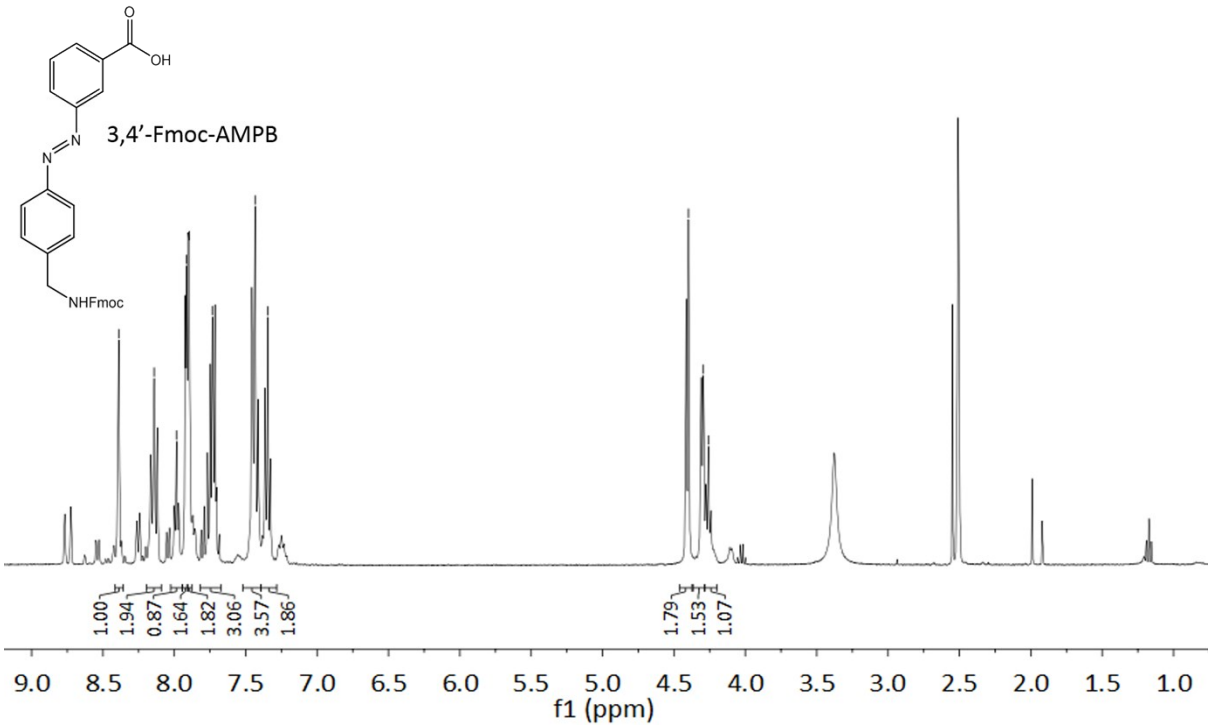
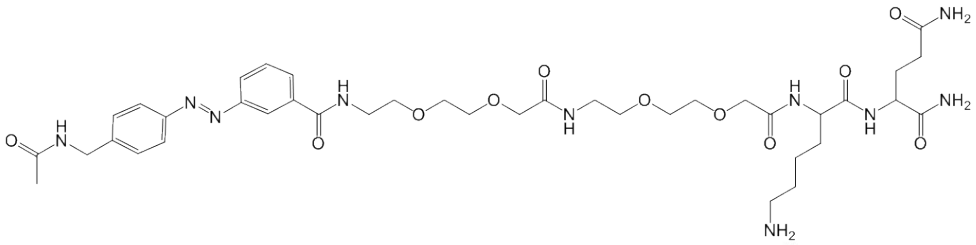


Fig. S2: ^{1}H NMR of 3,4’-AMPB in DMSO-d_6.

5. Guest Peptide

a)



b)

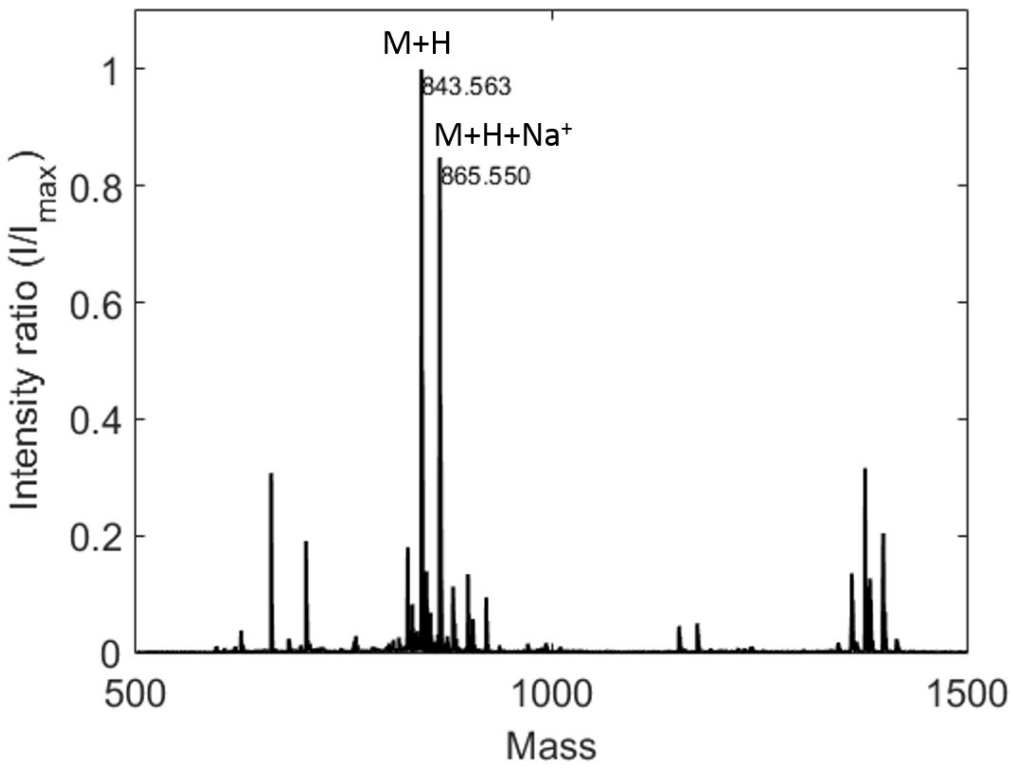


Fig. S3: a) ChemDraw structure of guest peptide. b) MALDI of guest peptide.

6. Thermal Relaxation of Azobenzene Guest Peptide

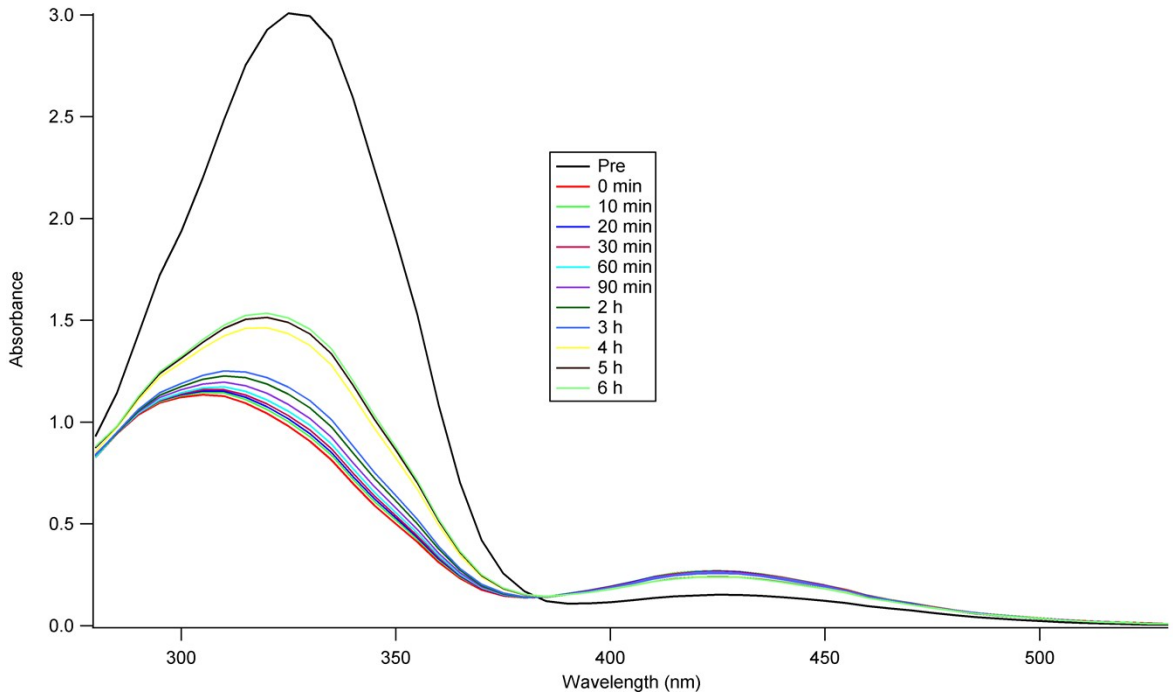


Fig. S4: UV-Vis absorbance spectra of guest peptide before UV induced *cis* isomerization and periodically thereafter. Used to compute half-life of thermal relaxation.

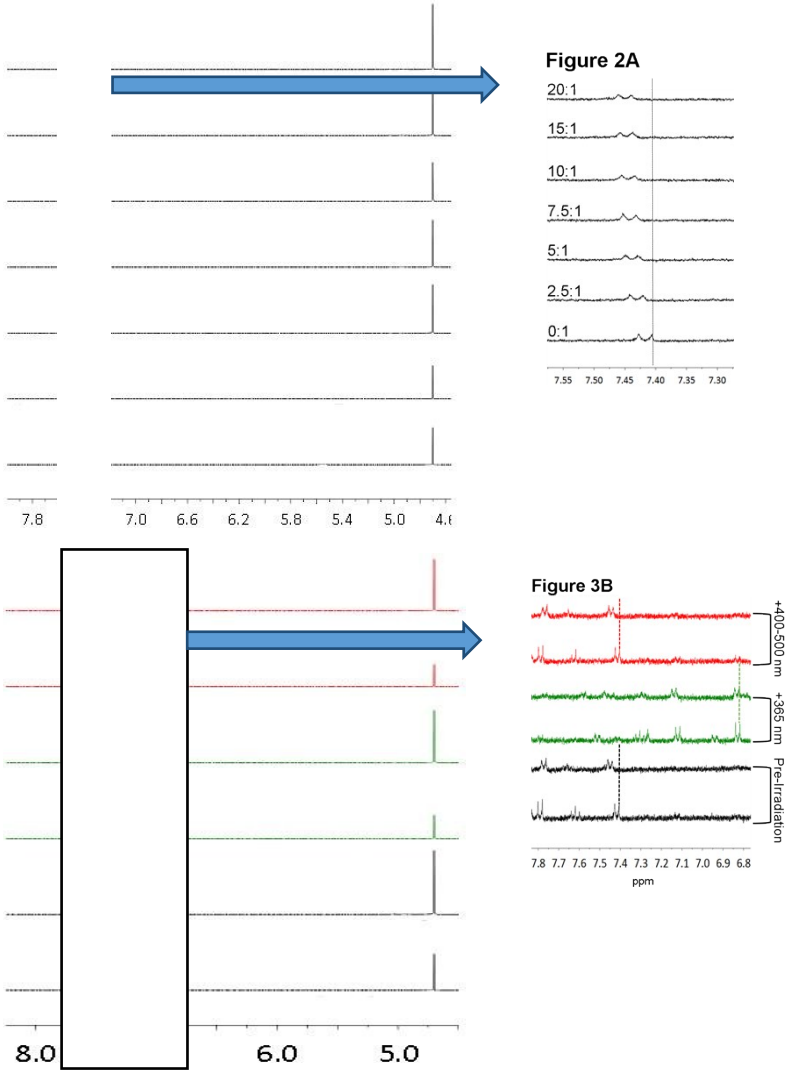


Fig. S5: D_2O reference peaks that correspond to the spectra in figures 2A and 3B.

7. NMR Reference Peaks

8. PEG-CD

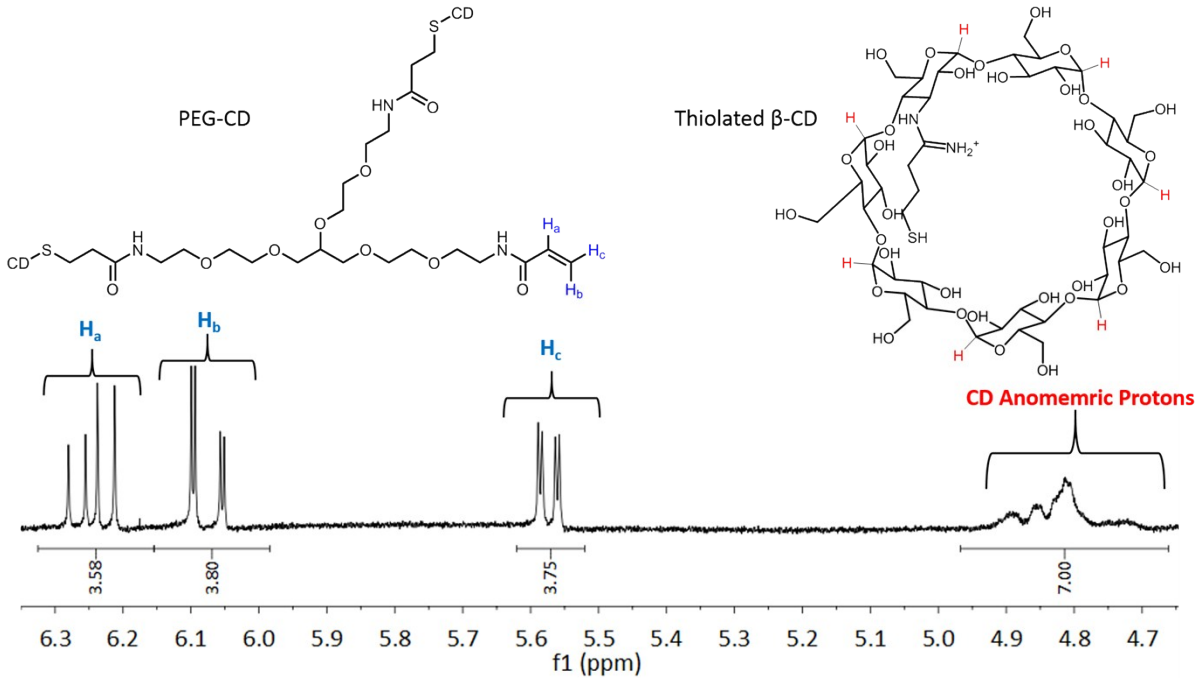


Fig. S6: Representative ^{1}H NMR spectrum of PEG-CD (in DMSO-d_6) with protons labeled and structures of PEG-CD and thiolated β-CD overlaid. Used to calculate the ratio of unmodified acrylamide groups to cyclodextrin moieties. Amino-3A-deoxy-β cyclodextrin

9. CD Free Control Gel

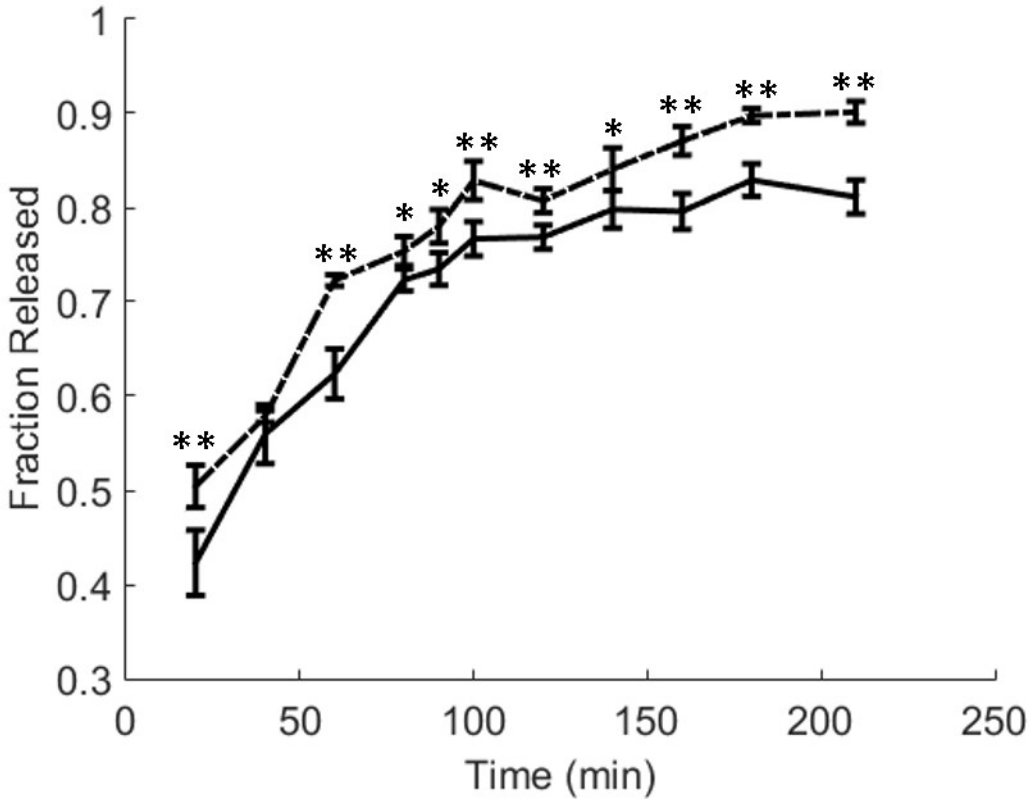


Fig. S7: Release profiles of an AF647 functionalized guest peptide from a CD free control gel (dashed) and a CD containing gel left at ambient lab conditions (solid). Asterisks denote statistical significance for p = 0.1 (single asterisk) and p = 0.05 (double asterisk) as determined by a one tailed t-test.

10. References

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