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Strong supramolecular control over protein self-assembly using a polyamine decorated β-cyclodextrin as synthetic recognition element

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Supporting information

General experimental

Reactions were performed at room temperature unless otherwise stated. Dry DMSO was purchased from Acros. Absolute EtOH, HPLC grade trifluoroacetic acid (TFA) and acetonitrile (MeCN) were purchased from Biosolve. All organic solvents were used as purchased. H_2O refers to Millipore grade distilled water. LC-MS analysis of ligated proteins: reverse-phase liquid chromatography-mass spectrometry (LC-MS) was performed on an Applied Biosystems Single Quadrupole Electrospray Ioniyation Mass Spectrometer API-150EX in positive mode using a Jupiter C4-column 150 x 2.0 mm. Eluent conditions (CH_3CN/H_2O/1% formic acid): 0-2 min, isocratic, 5 % CH_3CN; 2-10 min, linear gradient, 5 – 70 %; 10-12 min, isocratic, 70 %; 12-15 min, linear gradient, 70 – 5 %. LC-MS analysis of cyclodextrin **3**: Shimadzu LC Controller V2.0, LCQ Deca XP Mass Spectrometer V2.0, Alltima C18-column 125 x 2.0 mm, Surveyor AS and PDA. Eluent conditions (CH_3CN/H_2O/1% TFA): 0-1 min, isocratic, 2 % CH_3CN; 1-10 min, linear gradient, 2 - 10 %; 10-13 min, linear gradient, 10 – 70 %; 13-15 min, linear gradient, 70 – 2 %; 15-17 min, isocratic, 2 % CH_3CN. ^{1}H-NMR and ^{13}C-NMR spectra were recorded using a 400 MHz NMR (Varian Mercury). Proton chemical shifts are reported in ppm and calibrated with residual H_2O (4.79 ppm) as the internal standard. The splitting patterns are designated as: m, multiplet. Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra were obtained using a PerSeptive Biosystems Voyager-DE PRO spectrometer using an acid α-cyanohydroxycinnamic acid (CHCA) matrix.

Chemical Synthesis

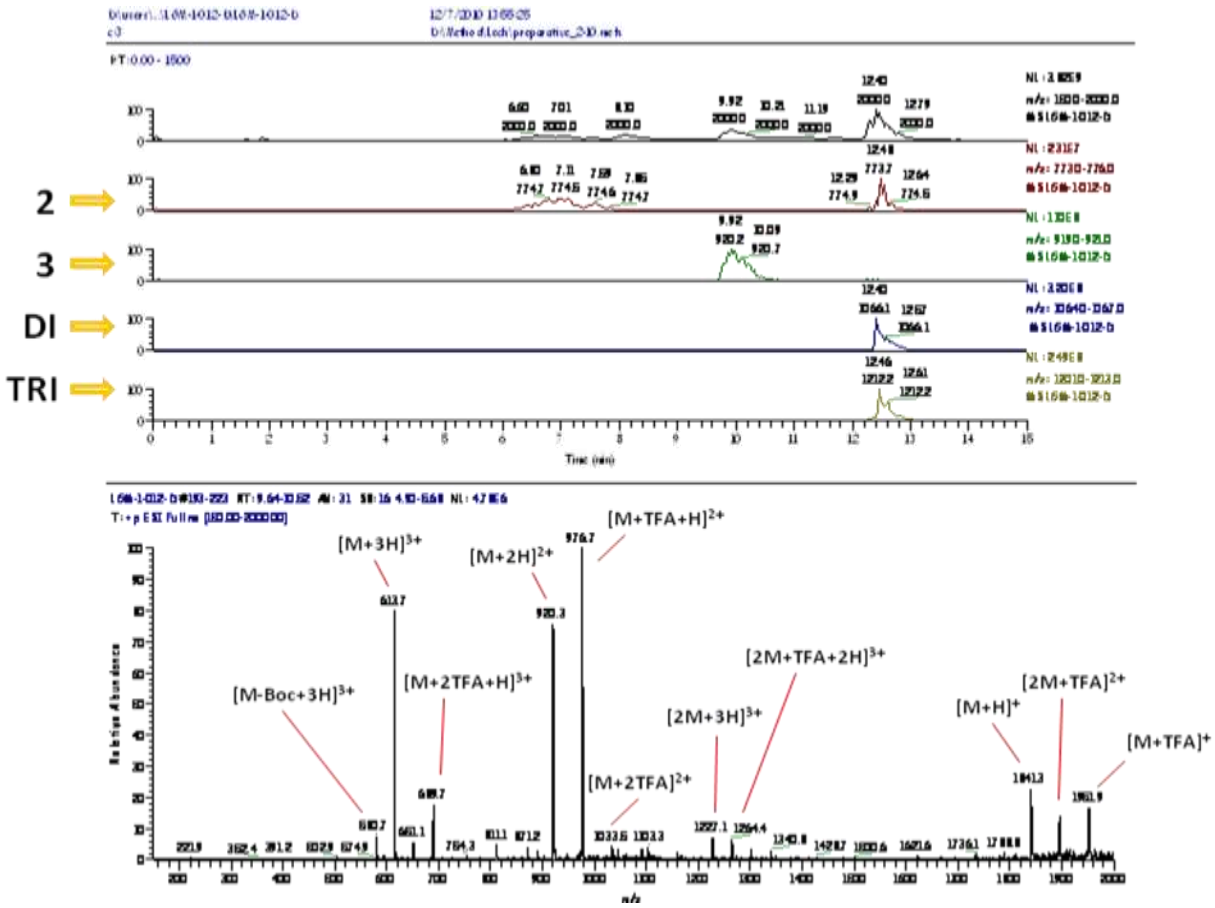
Mono-cysteine(Boc,StBu)-cysteamine-cyclodextrin 3

This reaction was performed under inert conditions using standard Schlenk line techniques. Dry triethylamine (1.25 mL, 8.97 mmol) was added drop-wise to a stirred soln. of crude cyclodextrin **2**[1] (430 mg, 0.238 mmol) in dry DMSO (8.5 mL). After 5 min, BocCysStBu-OSu[2], (96.7 mg, 0.238 mmol) was added drop-wise over 4 h via motorized syringe pump, and the reaction stirred for a further 22 h. The organic solvents were removed under high vacuum (45 °C, 1-2 mbar), to afford a clear faint-orange foam, which was suspended in EtOH (20 mL), stirred for 1 h, then isolated by suction filtration with thorough washing (EtOH, 6 x 5 mL). The solid crude material was re-dissolved in 10 mL H_2O and lyophilized to afford a pale-coloured solid (396 mg). A fraction of the total crude material (253 mg) was purified by reverse-phase preparative HPLC (Supporting Figures 1 and 2) with mass-triggered detection (Shimadzu LC Controller V2.0, LCQ Deca XP Mass Spectrometer V2.0, Alltima C18-column 125 x 20.0 mm): Eluent conditions (CH_3CN/H_2O/1% TFA): 0-1 min, isocratic, 2 % CH_3CN; 1-10 min, linear gradient, 2 – 10 %; 10-13 min, linear gradient, 10 – 70 %; 13-15 min, linear gradient, 70 – 2 %; 15-17 min, isocratic, 2 % CH_3CN; Collect mass peaks: 613.7 $[M+3H]^{3+}$, 920.6 $[M+2H]^{2+}$, 976.7 $[M+TFA+H]^{2+}$ between 9.5 and 10.5 min. Lyophilization of the combined fractions afforded the mono-functionalized cyclodextrin **3**, as the TFA salt (50.1 mg, MW = 2524.5, 19.8 µmol, 13%,[3] white powder). MALDI-TOF $C_{68}H_{126}N_8O_{31}S_9$ calculated 1838.6, found $[M+H]^+$ 1839.2. ^{1}H-NMR (400 MHz, D_2O) δ= 5.20-5.11 (m, CD), 4.38-4.32 (m, Cys), 4.20-2.80 (m), 1.53-1.40 (2 x *t*Bu).

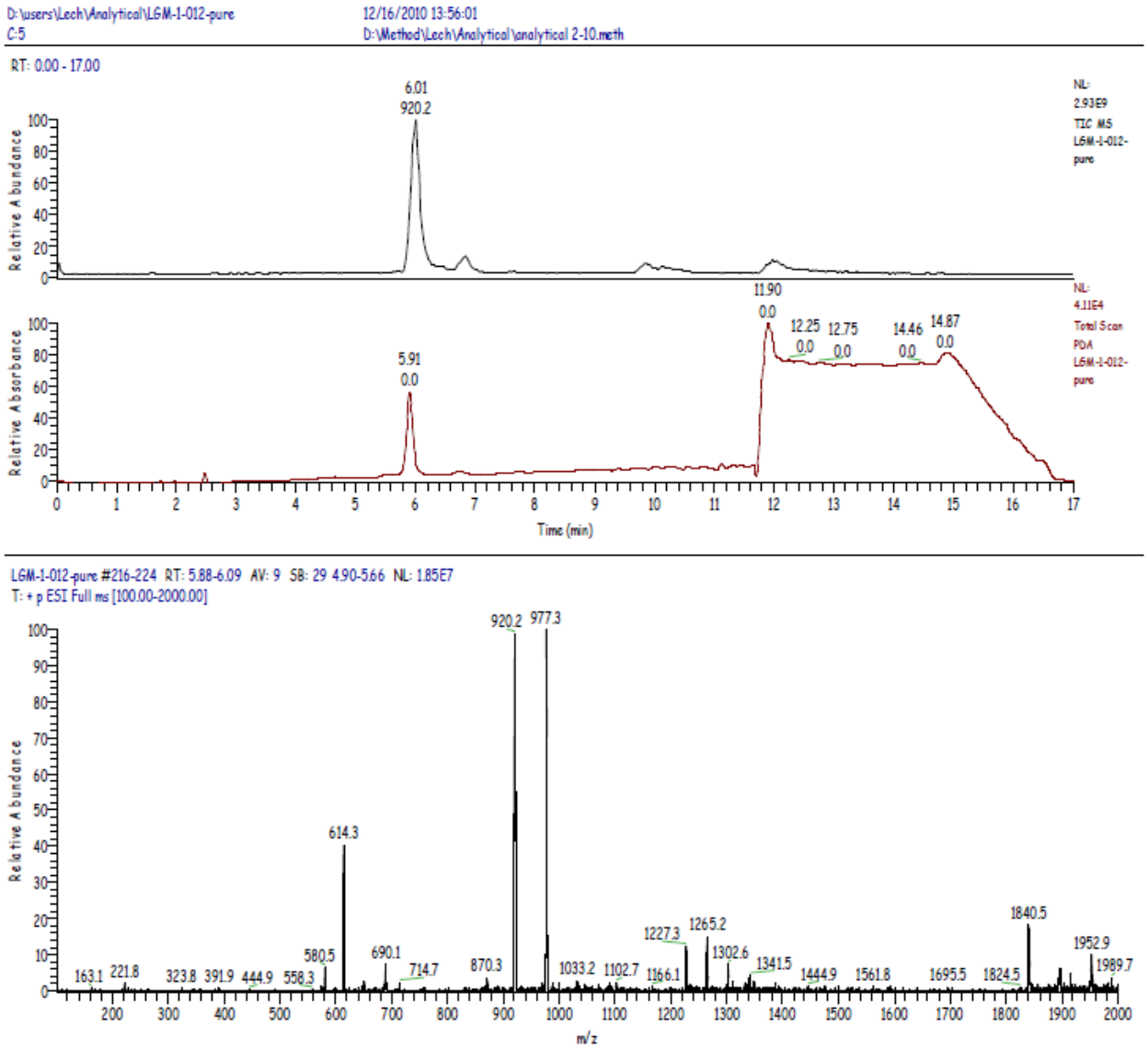
Mono-cysteine(StBu)-cysteamine-cyclodextrin 4

Boc-protected cyclodextrin TFA salt **3** (10.6 mg, 4.2 µmol) was stirred in 95% TFA/H_2O for 2 h 30 min. The solvents were then co-evaporated with 2 mL toluene *in vacuo* (repeated twice further) to afford a solid white paste, which was subsequently re-dissolved in 2 mL H_2O and then lyophilized to deliver the target cyclodextrin TFA salt, **4**, in sufficient purity for use in the planned protein ligation experiments (10,3 mg, MW = 2536.5, 97%, white powder). MALDI-TOF $C_{63}H_{118}N_8O_{29}S_9$ calculated 1738.6, found $[M+H]^+$ 1739.6, $[M+Na]^+$ 1761.5. ^{1}H-NMR (400 MHz, D_2O) δ= 5.18-5.09 (m, CD), 4.28-4.23 (m, Cys), 4.02-2.85 (m), 1.48-1.37 (m, 1 x *t*Bu).

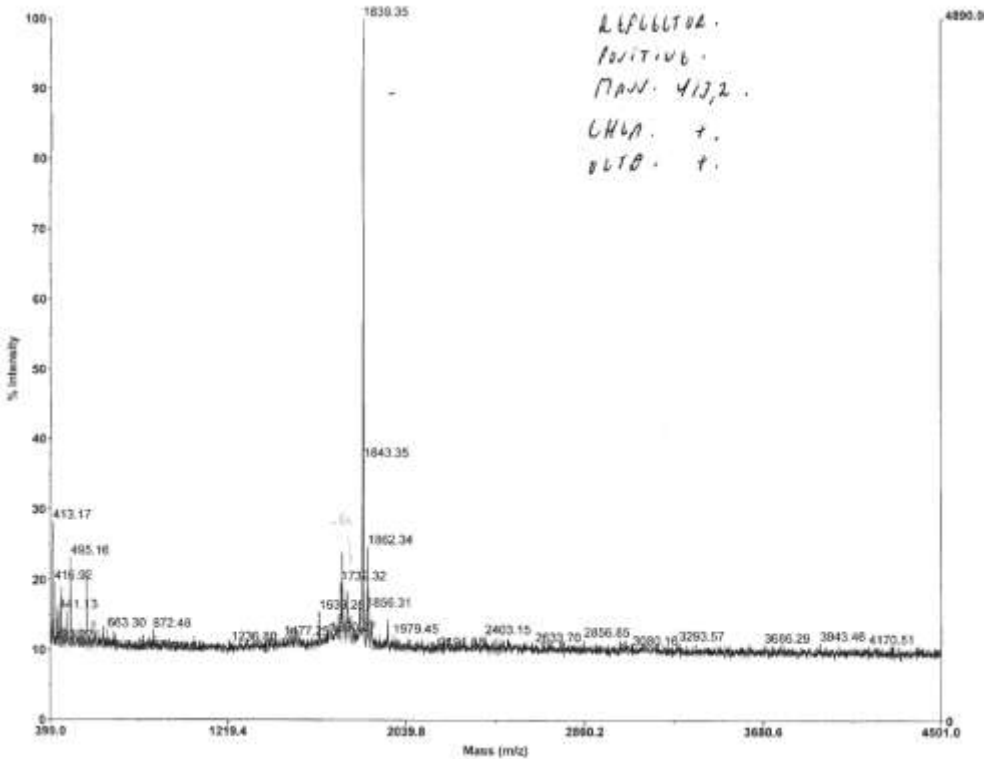
Analytical data



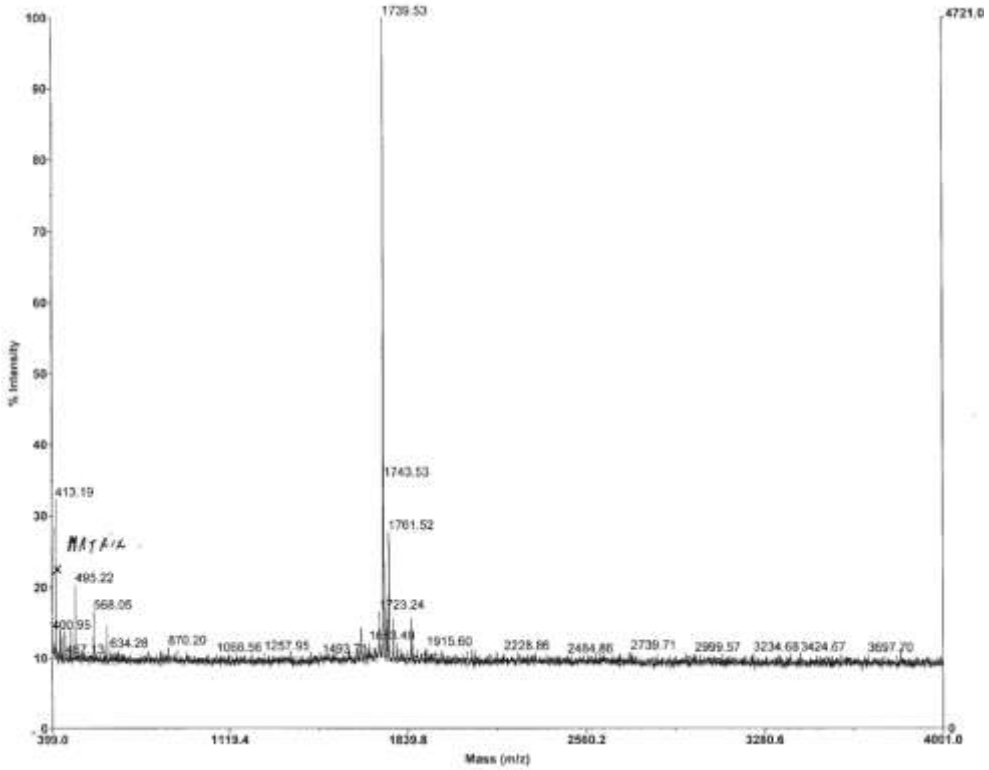
Supporting Figure 1. Preparative MS chromatogram (Alltima HP C18, 5 µM, 125 mm length, ID 20 mm) of **3** during purification. Above: Total Ion Count and masses corresponding to non-functionalized (**2**), mono-functionalized (**3**), and di- and tri-functionalized cysteamine-cyclodextrin (DI, TRI). Below: mass spectrum of the product peak 9.64-10.52 min.



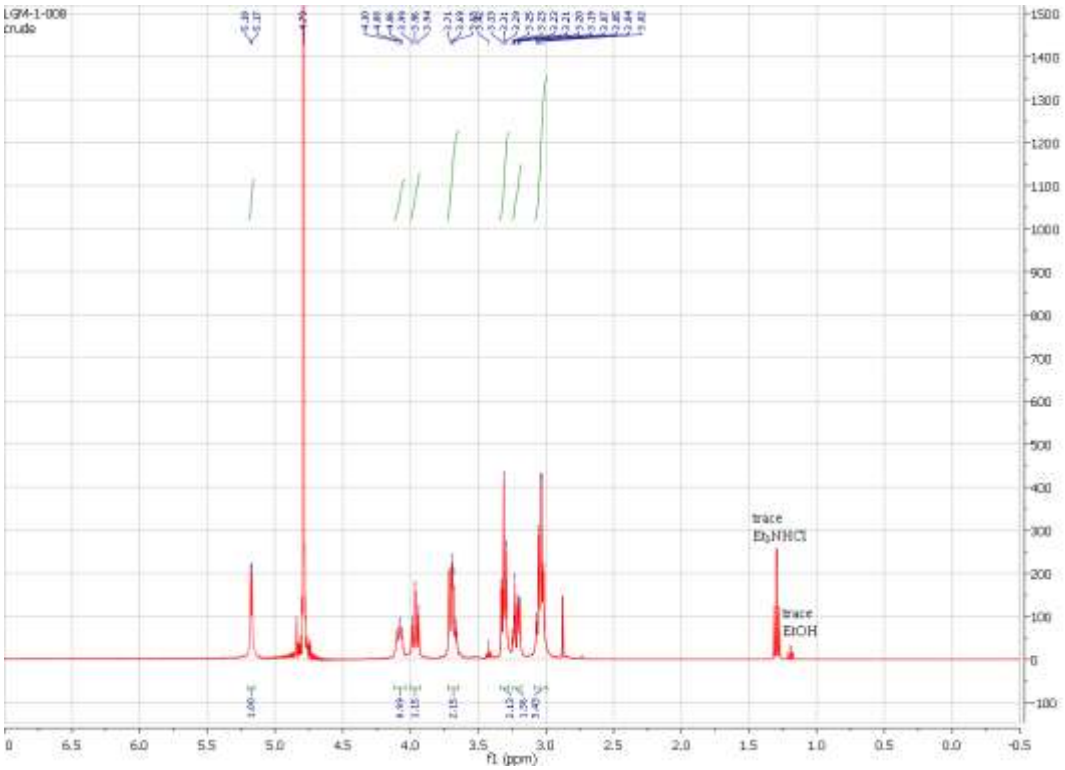
Supporting Figure 2. Analytical LC-MS spectrum (Alltima HP C18, 5 µM, 125 mm length, ID 2 mm) of **3** after purification. Above: TIC (top) and UV (bottom) after purification; Below: mass trace of the product peak 5.88-6.09 min.



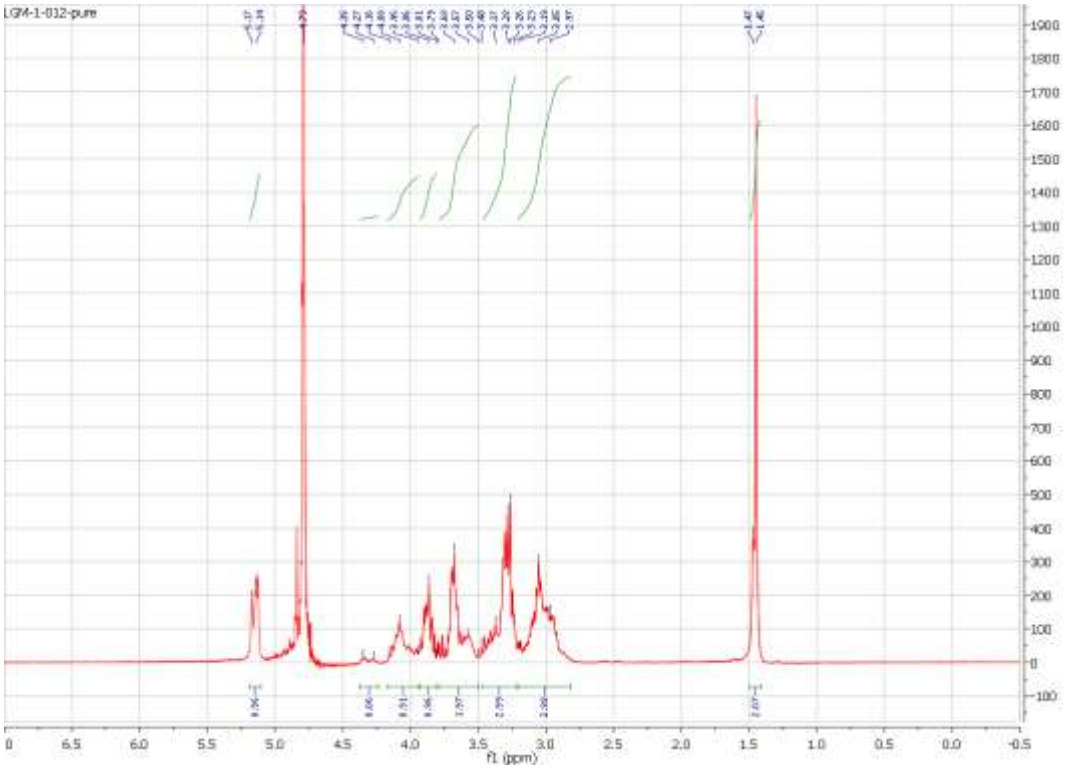
Supporting Figure 3. MALDI-TOF spectrum of mono-functionalized cysteamine cyclodextrin **3**.



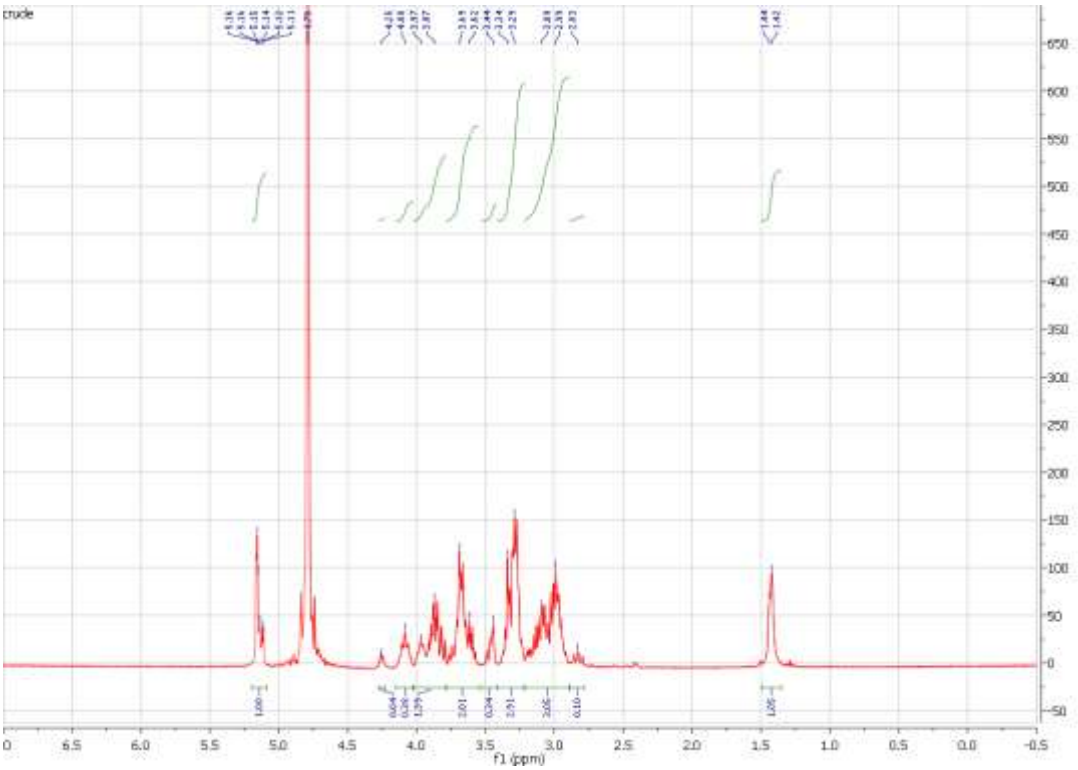
Supporting Figure 4. MALDI-TOF spectrum of mono-functionalized cysteamine cyclodextrin **4**.



Supporting Figure 5. ^{1}H-NMR of crude cysteamine-cyclodextrin (**2**) in D_2O.[4,5]



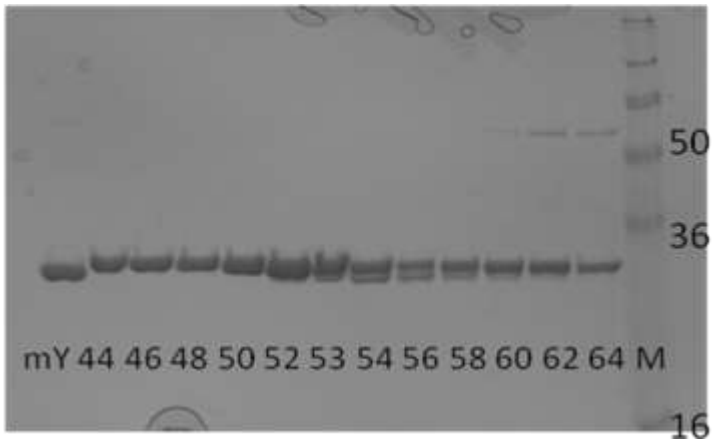
Supporting Figure 6. ^{1}H-NMR of pure Boc-protected mono-functionalized cysteamine-cyclodextrin (**3**) in D_2O.



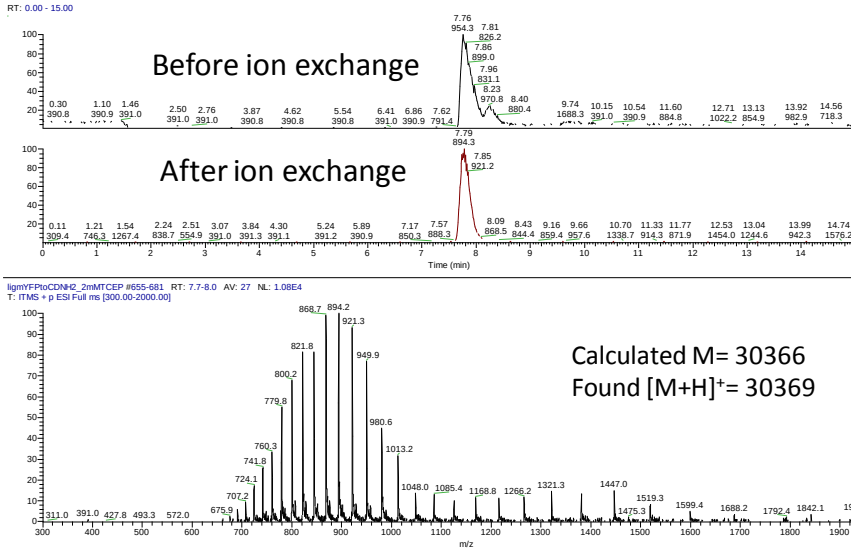
Supporting Figure 7. ^{1}H-NMR of mono-functionalized cysteamine-cyclodextrin (**4**) in D_2O.

Protein Ligation

An 8 mM solution of **4** in ligation buffer (25 mM Na-Pi, pH 7.5, 50 mM NaCl, degassed by bubbling Ar through the buffer) with 40 mM TCEP was prepared and incubated for 30 min to remove the -StBu protection group of **4**. This solution was added to the protein thioester (final concentration 210 µM) together with 4-mercapto-phenylacetic acid (30 mM) obtaining a final concentration of 2.7 mM of **6**. After incubation overnight approximately 60% of the protein (estimation by LC-MS) had reacted with compound **4**, and the remaining 40% were still present in the thioester form. To ensure complete modification of the protein, a further 5 eq. of deprotected cyclodextrin were added to the protein and incubated for 5 h. The excess of ligand, TCEP and MPAA were then removed by exchanging the buffer (20 mM TrisHCl pH 8, 50 mM NaCl) several times using centrifugal filters with a nominal molecular weight cut-off of 10 kDa. The protein solution was loaded manually onto a 1 mL HiTrap Q HP column (Q-sepharose $-CH_2N^+(CH_3)_3$) and then inserted into a Aekta Prime FPLC system. After washing with 20 column volumes of buffer A (20 mM TrisHCl pH 8, 50 mM NaCl), a linear gradient with a maximal concentration of 50% of buffer B (20 mM TrisHCl pH 8, 1 M NaCl) was applied (flow rate 1 mL/min, fraction size 0.2 mL). Elution of the protein started at a concentration of approximately 20% B. The fractions were analysed by SDS-PAGE and LC/MS. The pure fractions were pooled and after buffer exchange with buffer A, the protein was stored in aliquots at a concentration of 6 mg/mL at -80 °C. In total 1.5 mg (60 %) of mYCDNH$_2$ were isolated. LC-MS R_t= 7.8 min, $C_{1352}H_{2080}N_{346}O_{415}S_{17}$ calculated M= 30366, found 30369.



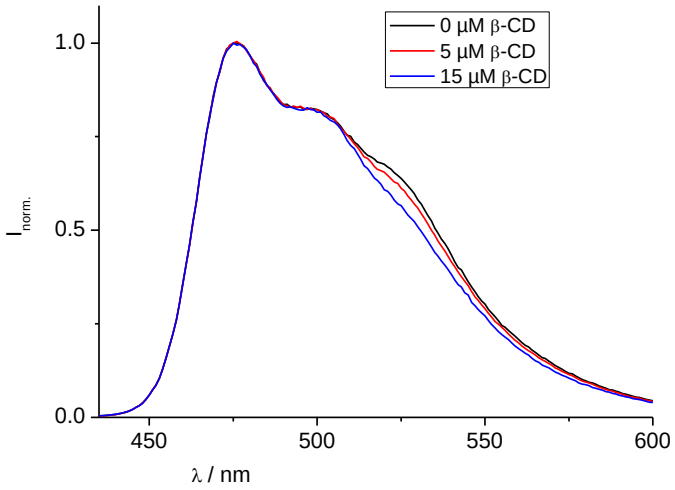
Supporting Figure 8. SDS-PAGE, fractions of ion-exchange chromatography of mYFPPCDNH$_2$, pooled fractions 44-51. mY is the starting mYFP protein thioester. M is the molecular weight marker.



Supporting Figure 9. Top: LC/MS spectra of mYCDNH$_2$ before and after ion exchange chromatography (TIC signal); Bottom: MS spectrum of the purified protein after ion exchange with its deconvoluted mass.

Fluorescence Spectroscopy

All samples for fluorescence spectroscopy measurements were prepared under ambient conditions in quartz cuvettes. Samples were prepared in sodium phosphate buffer (25 mM sodium phosphate, 50 mM NaCl, pH 7.5) with 1 mM TCEP. The concentration of the proteins was determined by UV/Vis spectroscopy on a NanoDrop ND-1000 spectrophotometer using the absorbance at 435 nm and ε_{435}= 32500 $M^{-1}cm^{-1}$ for mCFP and the absorbance at 515 nm and ε_{515}= 84000 $M^{-1}cm^{-1}$ for mYFP. Fluorescence data were recorded on a Varian Cary Eclipse photoluminescence spectrometer. Fluorescence emission spectra were recorded at an excitation wavelength of 410 nm.



Supporting Figure 10. Fluorescence emission spectra of 0.5 µM protein complex mYCD-mCLA (black) after addition of 5 µM (red) and 15 µM β-Cyclodextrin.

References

[1] M. Gómez-García, J. M. Benito, D. Rodríguez-Lucena, J.-X. Yu, K. Chmurski, C. Ortiz Mellet, R. Gutiérrez Gallego, A. M., J. Defaye, J. M. García Fernández, *J. Am. Chem. Soc*., **2005**, *127*, 22, 7970–7971.
[2] R. Jaouhari, T. Besheya, J.H. McKie, K.T. Douglas, *Amino Acids* **1995**, *9*, 327-342.
[3] In proportion with the quantity of crude material purified.
[4] M. Gómez-García, J. M. Benito, D. Rodríguez-Lucena, J.-X. Yu, K. Chmurski, C. Ortiz Mellet, R. Gutiérrez Gallego, A. M., J. Defaye and J. M. García Fernández, *J. Am. Chem. Soc*., 2005, **127**, 7970–7971.
[5] R. F. Gomez-Biagi, R. B. C. Jagt and M. Nitz, *Org. Biomol. Chem*., 2008, **6**, 4622–4626.