Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Polymer-peptide chimeras for the multivalent display of immunogenic peptides

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Materials and Methods

N-isopropylacrylamide (NiPAAM) (Sigma Aldrich, 99%) was purified by recrystallisation from hexane (Ajax) three times and dried under vacuum at room temperature before use. Azobis(isobutyronitrile) (AIBN) was purified by recrystallisation from methanol twice and then left to dry under reduced pressure. 1,4 Dioxane (Sigma Aldrich, 99%) was distilled and stored under nitrogen prior to use. Trithiocarbonate RAFT agent, 2-(butylthiocarbonothioylthio)propanoic acid (C4-RAFT) (Figure 1) was synthesised in accordance with a previously published protocol.[1] Trimethylsilyl-2-propyn-1-ol (TMSP) was synthesised as described in the literature.[2] Dimethylformamide (DMF) stored over molecular sieves (Labscan, 99%, anhydrous, low amine content) was used as received. Anhydrous dichloromethane (DCM), methanol, anhydrous tetrahydrofuran (THF), tetra-*n*-butylammonium fluoride (TBAF), pyridine, tris[2-carboxyethyl]phosphine hydrochloride (TCEP), sodium ascorbate, sodium borohydride, *N*-(1-Pyrenyl)maleimide, copper sulfate pentahydrate, calcium sulfate, copper bromide, *n*-methyl morpholine, n,n,n',n',n-pentamethyldiethylenetriamine (PMDETA), triisopropylsilane (TIPS), thioanisole and *N*-Diisopropylcarbodiimide were purchased from Sigma Aldrich and used as received. Amino acids and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) for peptide synthesis were purchased from Novabiochem and used without purification. Acetic anhydride, trifluoroacetic acid, acetic acid and hydrochloric acid were purchased from Ajax fine chemicals and used as received. Tetra-*n*-butylammonium bromide was obtained from Merck and used as received. Purification *via* dialysis was carried out using Spectra Por dialysis tubing with MWCO 2000 Daltons.

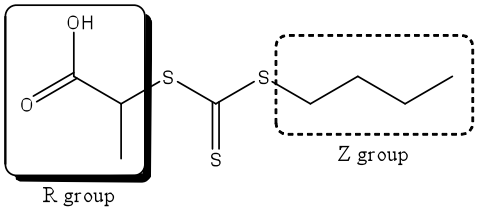


Figure S1. 2-(butylthiocarbonothioylthio)propanoic acid (C4-RAFT) chain transfer agent.

Nuclear Magnetic Resonace (NMR). NMR analyses were carried out on Bruker Ultra Shield Avance spectrometers (300 or 200 MHz). For all NMR analyses, unless stated otherwise, deuterated chloroform ($CDCl_3$) was used as the solvent with tetramethylsilane (TMS) as the internal standard.

Size exclusion chromatography (SEC). SEC analyses were carried out at 40 ^{o}C using a Shimadzu SEC system equipped with a guard column and two Polymer Laboratories PLgel 10 μm Mixed-B columns (molecular weight range of 500-10 000 000g/mol) attached to a differential refractive index (DRI) detector (Shimadzu, RID-10A) and a UV-Vis detector (Shimadzu, SPD-10A VP). THF with tetra-*n*-butylammonium bromide (0.25% w/v) was used as the eluent and the flow rate was set at 1ml/min. The system was calibrated using Polymer Laboratories narrow molecular weight distribution polystyrene standards.

Dynamic Light scattering (DLS). Particle size measurements were carried out by dynamic light scattering (DLS) using a Malvern Instruments Zetasizer nano series instrument. An equilibration time of 3 minutes (minimum) was allowed before each measurement and at least five replicate measurements were made for each sample.

Transmission electron microscopy (TEM). Samples were prepared by placing a drop of sample onto a carbon coated copper grid followed by addition of a drop of the staining solution (uranyl acetate). Excess solution was carefully blotted off using filter paper and samples were air dried for at least 10 minutes under a UV lamp before analysis. TEM images were obtained using a Philips CM120 electron microscope.

Fourier transform infra-red (FT-IR). FT-IR spectra were recorded using a Perkin Elmer Spectrum-One FT-IR spectrometer. The number of scans per sample was set at 100.

Fluorescence and UV-Vis Spectroscopy. Fluorescence spectroscopy analyses were conducted using a Cary Eclipse fluorescence spectrometer and UV-Vis measurements were carried out using a Cary 50 Bio UV-visible spectrometer.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). MALDI-TOF mass spectrometry experiments were undertaken

using a Waters (Micromass) TOF SPEC 2E mass spectrometer equipped with a nitrogen laser (λ = 337 nm). The accelerating voltage was 20 kV. Samples were dissolved in methanol at a concentration of 1mg/ml. The spectra were obtained in positive mode and the matrix employed was α-Cyano-3-hydroxycinnamic acid. Sample and matrix were mixed and left to dry on a stainless steel plate. Data collection and analysis was carried out using MassLynx software.

Electrospray ionisation mass spectrometry. Mass Spectrometry was conducted using a Finnigan LCQ Deca MS detector with XCalibar Data Processing and Instrument Control Software. Samples of appropriate concentration were made up in methanol before injection into the electrospray ionization unit at 0.2 mL min^{-1}. The electrospray voltage was 5 kV, the sheathing gas was nitrogen at 415 kPa, and the heated capillary was set at 200 °C.

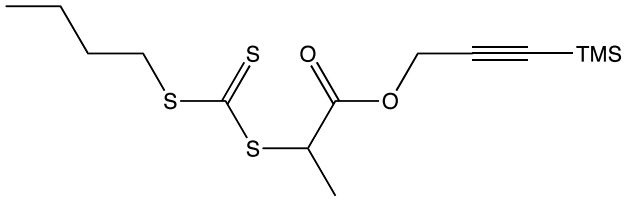
Reverse Phase High Performance Liquid Chromatography (RP-HPLC). Analytical reverse-phase (RP-) HPLC was performed on a Waters System 2695 separations module with an Alliance series column heater at 30 °C and 2996 photodiode array detector and employed a Waters Sunfire C18 column (2.1 x 150 mm column, 5 μm particle size, flow rate of 0.2 mL min^{-1}). Preparative RP-HPLC was performed using a Waters 600 Multisolvent Delivery System and Waters 500 pump with a 2996 photodiode array detector or Waters 490E Programmable wavelength detector operating at λ = 230 employing a Waters Sunfire Prep C18 OBD column (19 x 150 mm, 5 μm particle size, flow rate 7 mL min^{-1}). The mobile phase consisted of eluents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) for all HPLC runs.

pH meter. Measurements were conducted at 27 ± 1 ^{o}C using a calibrated HANN instruments pH meter.

DSC measurements. DSC measurements were undertaken using a TA Instruments modulated DSC 2920 calibrated using an indium metal standard. The heating rate was 1 ^{o}C per minute and the temperature was held constant at the low extreme temperature for 10 minutes before measurement.

Experimental

3-(trimethylsilyl)prop-2-ynyl2-(butylthiocarbonothioylthio) propanoate alkyne functionalised RAFT agent (3).



To a dry round bottomed flask was added C4 RAFT agent (2.0 g, 8.4 mmol) and 3-TMSP (3.6 g, 28.1 mmol). DCM (100 mL) at 0 ^{o}C was added to the flask with swirling to ensure complete dissolution of reactants. The round bottomed flask was placed in an ice bath which was positioned on a magnetic stirrer and the flask contents were left to stir for 10 minutes. 4-dimethylaminopyridine (1.0 g, 8.4 mmol) and *n*-(3-dimethylaminopropyl)-n'-ethylcarbodiimide hydrochloride (3.7 g, 25.2 mmol) dissolved in a minimal amount of DCM were then slowly added to the round bottomed flask *via* a pressure equalising dropping funnel. The reaction was stirred for a further 4 h at 0 ^{o}C and at room temperature for a further 16 hours. The reaction mixture was then washed with water (5 x 100 mL), dried over $MgSO_4$ and the solvent removed *in vacuo*.. Purification of the crude product by flash chromatography (eluent: 9:1 v/v hexane/ethyl acetate) gave **3** as a yellow oil. (1.6 g, 55%). ^{1}H NMR (300 MHz, $CDCl_3$): 0.2 (s, 9H), 0.95 (t, *J*=7.32 Hz, 3H), 1.45 (sextet, *J*=7.55 Hz, 2H), 1.58 (d, *J*=7.38 Hz, 3H), 1.7 (p, 2H), 3.37 (t, *J*=7.38 Hz, 2H), 4.73 (s, 2H), 4.85 (q, *J*=7.36 Hz, 1H). ESI MS m/z = 371 $(M+Na)^{+}$

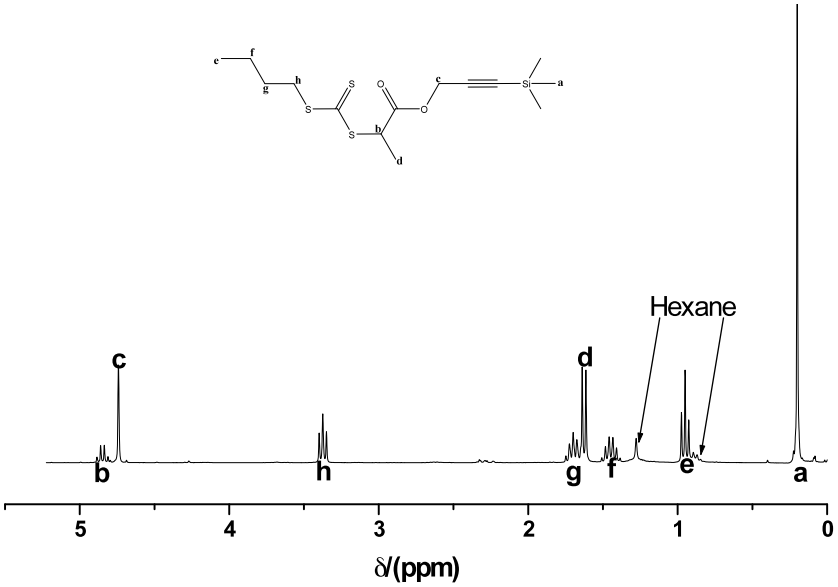


Figure S2. ^{1}H NMR of purified 3-(trimetylsilyl)prop-2-ynyl2-(butylthiocarbonothioylthio) propanoate alkyne (3).

Polymerisation of NiPAAM (Typical procedure). C4 RAFT-Alkyne **3** (0.130g, 0.35 mmol) and AIBN (0.006g, 0.04 mmol) were weighed separately into glass vials and dissolved in dioxane (1.0 mL). The RAFT agent and initiator solutions were then added to a pre-weighed amount of NiPAAM in a vial containing a stirrer bar. An additional 1.5 mL of dioxane was added to the NiPAAM (1.000g, 8.82 mmol) to obtain a homogeneous solution. The vial was sealed using a suba seal and oxygen was removed from the solution by sparging with nitrogen for 15 minutes. After degassing, the vial was transferred to a heated oil bath maintained at 60 ^{o}C and polymerisation was allowed to proceed for 150 minutes. The reaction was stopped by placing the vial in an ice bath and opening the reaction to the atmosphere. Conversion was determined by ^{1}H NMR. Purification of the polymers was achieved *via* repeated precipitation in cold hexane/diethyl ether solution (1:4 v/v). Conversion: 80%; M_n = 2600 g/mol, *PDI* = 1.2 (SEC).

TMS deprotection (General procedure). Silyl deprotection was carried using a modified procedure reported by Ladmiral *et al*.[3] Protected PNiPAAM (300 mg) was dissolved in tetrahydrofuran (10 mL) and the vial purged with nitrogen for 10 minutes. Acetic acid (10 mol%) was added and the solution was cooled to -20 ^{o}C.

TBAF in THF (10 eq.) was added dropwise to the polymer solution and the reaction mixture was stirred at -20 ^{o}C for 30 minutes followed by stirring at rt for 16 hours. The reaction mixture was passed through a short silica column to remove TBAF and the polymer solution was concentrated *in vacuo*. Purification of the polymers was achieved *via* repeated precipitation in cold hexane/diethyl ether solution (1:4 v/v).

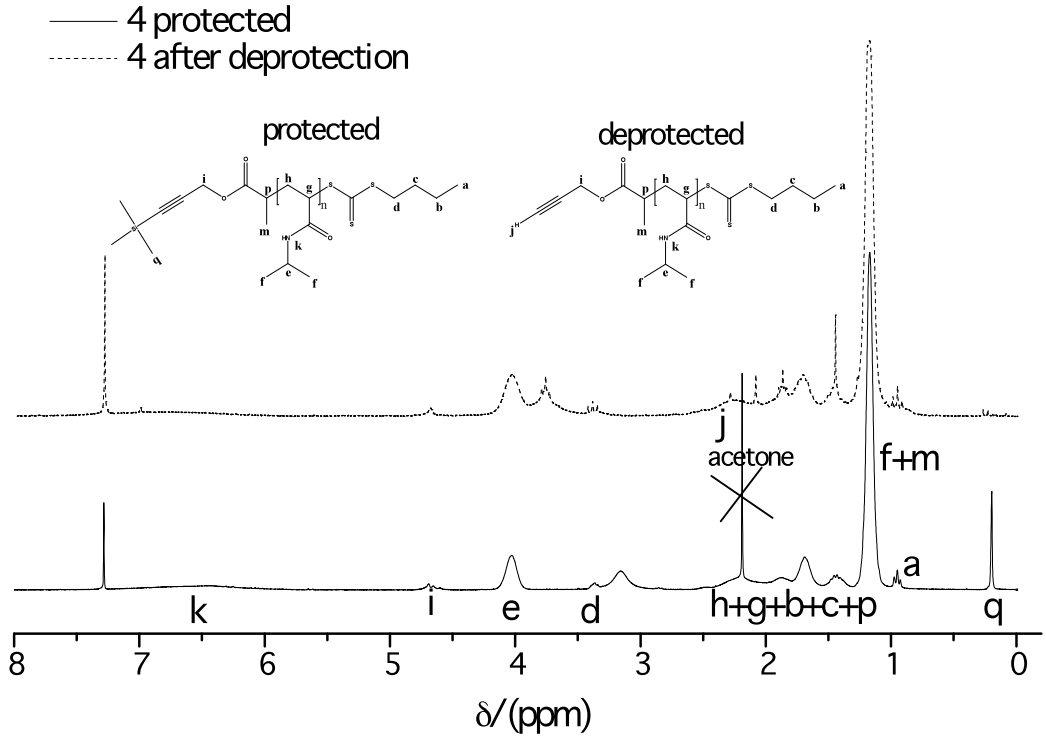


Figure S3. ^{1}H NMR of purified 4 before and 4 after deprotection with TBAF

Synthesis of MUC1 azidopeptides 1 and 2.

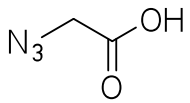
Pre-loading Wang resin

Wang resin was swelled in DMF for 30 min before use. Fmoc-Ala-OH or Fmoc-His(Trt)-OH (8 eq.) was dissolved in anhydrous DCM (final concentration 0.1 M) and cooled to 0 ^{o}C. *N,N*-Diisopropylcarbodiimide (4 eq.) was added dropwise and the reaction stirred for 30 min at rt. The reaction mixture was concentrated *in vacuo* and dissolved in DMF (final concentration 0.1 M) containing DMAP (0.1 eq.). This was added immediately to the pre-swelled Wang resin and placed on a shaker for 30 min. The resin was washed with DMF (× 5), DCM (× 5) and DMF (× 5) before capping with acetic anhydride/pyridine solution 1:9 v/v followed by washing with DMF (× 5), DCM (× 5) and DMF (× 5). Resin loading was shown to be quantitative, as

determined by deprotecting with 10% piperidine in DMF (2 × 3 min) and measuring the absorbance of piperidine-fulvene adduct at λ = 301 nm.

Iterative peptide assembly (Fmoc-strategy): *Deprotection:* The resin was treated with 10% piperidine/DMF (2 × 3 min) and washed with DMF (× 5), DCM (× 5) and DMF (× 5). *Amino acid coupling:* A pre-activated solution of protected amino acid (4 eq.), PyBOP (4 eq.) and NMM (8 eq.) in DMF (final concentration 0.1 M) was added to the resin. After shaking for 1 h, the resin was washed with DMF (× 5), DCM (× 5) and DMF (x 5). *Azidoglycine coupling:* A pre-activated solution of azidoglycine (4 eq.), PyBOP (4 eq.) and NMM (8 eq.) in DMF (final concentration 0.1 M) was added to the resin. After shaking for 1 h, the resin was washed with DMF (× 5) and DCM (× 10) and the capping step omitted. *Cleavage*: A mixture of TFA, triisopropylsilane (TIS) and water (90:5:5 v/v/v) was added to the resin. After shaking for 1.5 h, the resin was washed with TFA (3 × 2 mL) *Work-up*: The combined cleavage solution and TFA washings were concentrated *in vacuo*. The residue was dissolved in water containing 0.1% TFA and purified by preparative HPLC (Gradient 0 to 25% B over 60 min).

Synthesis of azidoglycine



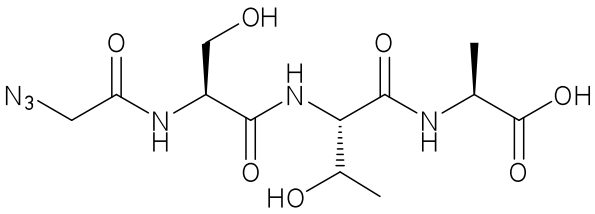
A solution of methyl bromoacetate (0.80 mL, 8.5 mmol) and sodium azide (0.60 g, 9.0 mmol, 1.1 eq.) in DMF (5 mL) was stirred at room temperature for 2.5 h. The reaction mixture was diluted with water (50 mL) and extracted with diethylether (4 x 10 mL). The combined organic fractions were washed with water (4 x 10 mL), dried (Na_2SO_4) and concentrated *in vacuo* to obtain azidoglycine methyl ester as a yellow oil (235 mg, 24%) which was used without further purification. Potassium hydroxide (0.3 g, 5.0 mmol) was dissolved in a solution of THF/H_2O (1:1 v/v, 8 mL) and added to azidoglycine methyl ester. The reaction was stirred at 40 ^oC for 2.5 h before washing with ethyl acetate (10 mL). The aqueous fraction was acidified to pH 1 with 3 M HCl, then extracted with ethyl acetate (4 x 20 mL) and the combined organic fractions dried over Na_2SO_4 and concentrated *in vacuo* to obtain azidoglycine as a yellow oil (154 mg, 75%).

ESI (m/z): $[M-H]^-$: 99.8; ^{1}H NMR ($CDCl_3$, 300 MHz): 9.36 (1H, br s, OH), 3.97 (2H, s, CH_2); ^{13}C NMR ($CDCl_3$, 50 MHz): 173.6, 50.0

These data are in agreement with that previously reported by Kim *et al.*[4]

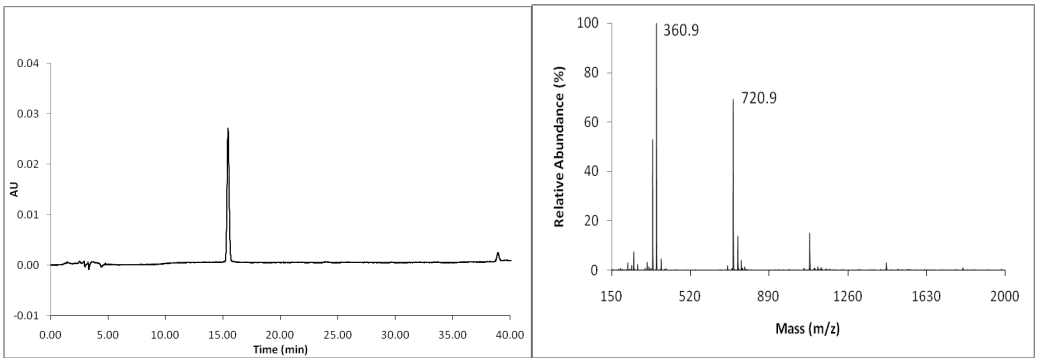
Tetrapeptide (1)

Tetrapeptide **1** was synthesised using the iterative peptide assembly described above.



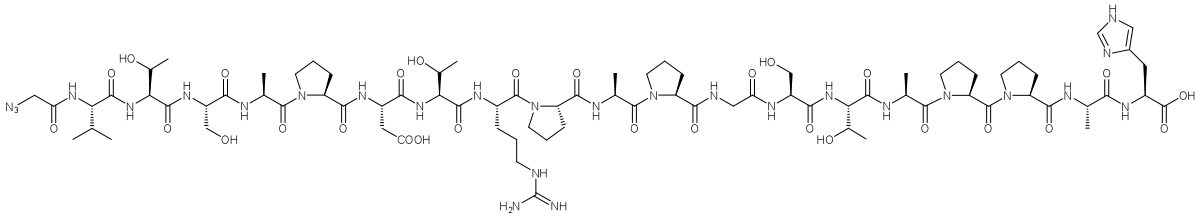
26 μM scale, Yield = 9.6 mg, quant., yield based on the Fmoc loading of the penultimate serine residue.

ν_{max}/cm^{-1} 3278, 3078, 2944, 2108, 1629, 1549; ESI (m/z): $[M+H]^+$: 360.9, $[2M+H]^+$: 720.9; HPLC: t_R: 15.5 min (Gradient 0 to 25% B over 40 min); ^{1}H NMR (MeOD, 400 MHz): 4.88 (1H, app. t, *J* 5.9 Hz, CH), 4.55-4.38 (2H, m, 2 × CH), 4.27-4.25 (1H, m, CH), 3.98 (2H, s, CH_2), 3.83 (1H, dd, *J* 5.5, 10.9 Hz, C*H*H), 3.77 (1H, dd, *J* 6.2, 10.9 Hz, CH*H*), 1.41 (3H, d, *J* 7.3 Hz, CH_3), 1.23 (3H, d, *J* 6.4 Hz, CH_3); ^{13}C NMR (MeOD, 100 MHz): 175.8, 172.5, 172.2, 170.4, 68.2, 62.9, 60.0, 56.6, 52.7, 49.5, 20.0, 17.6; HRMS calcd for $C_{12}H_{20}N_6O_7Na$: $[M+Na]^+$, 383.1286. Found: $[M+Na]^+$, 383.1286.



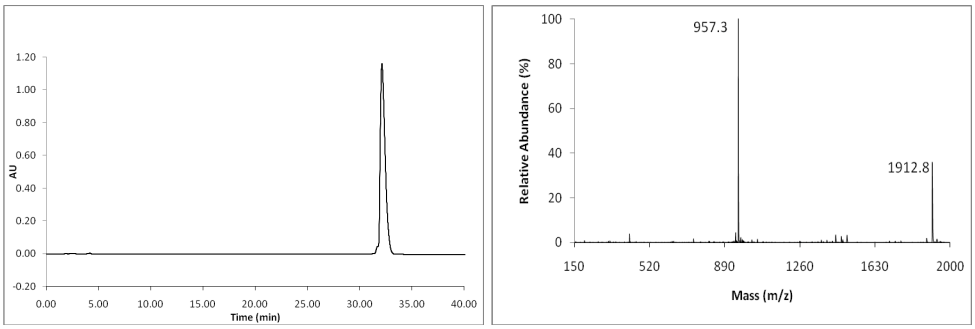
Eicosapeptide (2)

Eicosapeptide **2** was synthesised using the iterative peptide assembly described above.



17 μM scale, Yield = 31.9 mg, 98%, yield based on the Fmoc loading of the penultimate valine residue.

ν_{max}/cm^{-1} 3299, 2984, 2109, 1633, 1537, 1454; ESI (m/z): $[M+H]^+$: 1912.8, $[M+2H]^{2+}$ 957.3; HPLC: t_R: 32.2 min (Gradient 0 to 25% B over 40 min); HRMS calcd for $C_{80}H_{126}N_{27}O_{28}$: $[M+H]^+$, 1912.9260. Found: $[M+H]^+$, 1912.9202.



Typical polymer peptide click conjugate - PNiPAAM with eicosapeptide 2.

PNiPAAM (9.8 mg, 3.7 μmol, M_n (NMR) =2600 g/mol, PDI = 1.2) and eicosapeptide **2** (8.0 mg, 4.2 μmol) were dissolved in methanol (3 mL) in a dry vial and the solution was sparged with nitrogen for 15 minutes. In another vial, copper bromide (3 mg, 20.9 μmol) and PMDETA (3.7 mg, 21 μmol) were mixed in degassed methanol (2 mL) and the sealed vial was also purged with nitrogen for 15 min. The CuBr/PMDETA solution was then transferred into the polymer/peptide solution *via* cannula and the reaction was allowed to proceed for 60 hours. The reaction mixture was then passed through a short column of silica which was eluted using methanol. After concentration of the collected solution *in vacuo*, an aliquot was taken for analysis by ESI-MS and SEC DMF. The reaction progress was monitored by the disappearance of the limiting reagent (PNiPAAM) from the ESI spectrum. The polymer-peptide

conjugate was purified by dialysis against water for 5 days then analysed by FT-IR and ^{1}H NMR.

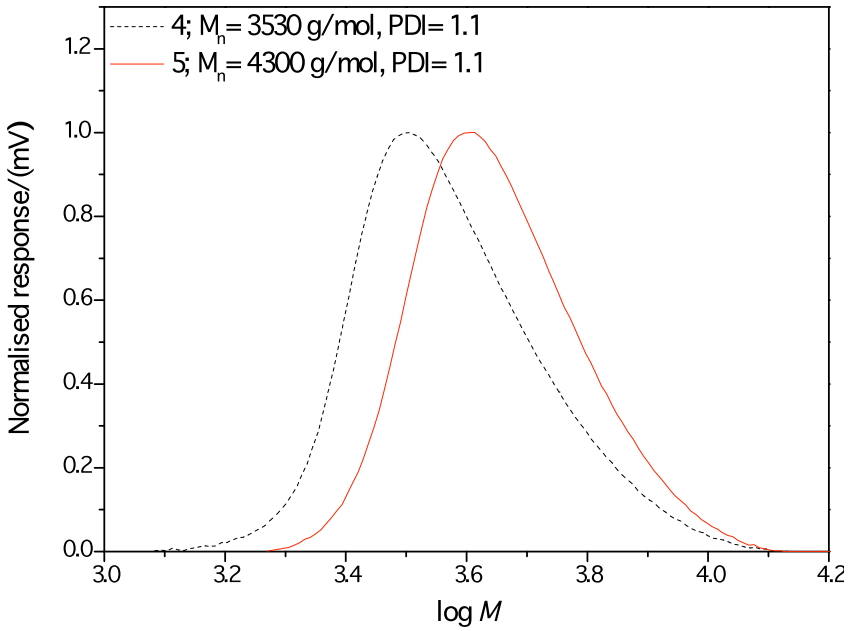


Figure S4. SEC THF chromatograms of PNiPAAM before and after conjugation of 1 *via* the Cu-catalysed alkyne-azide cycloaddition (CuAAC) reaction.

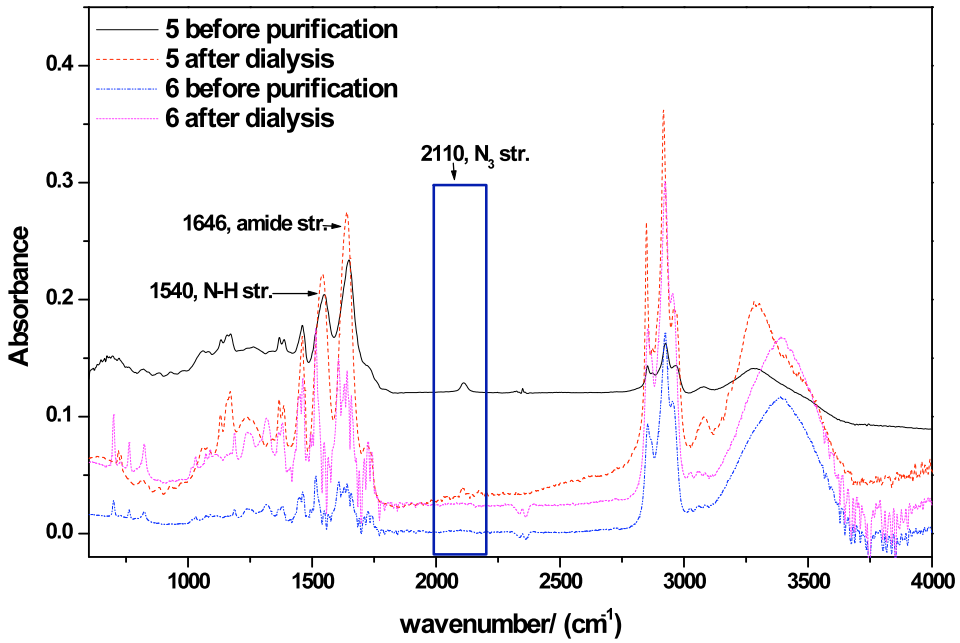


Figure S5. FT-IR spectra of polymer-peptide chimeras 5 and 6 before purification and after purification by dialysis.

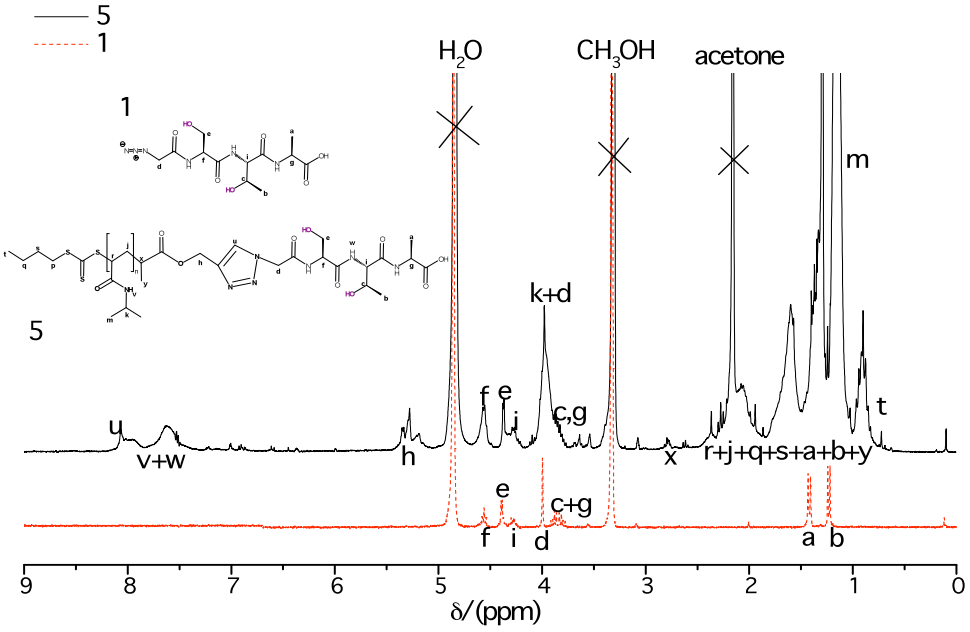


Figure S6. ^{1}H NMR spectra of 1 (bottom) and 5 obtained after conjugation *via* the CuAAC reaction.

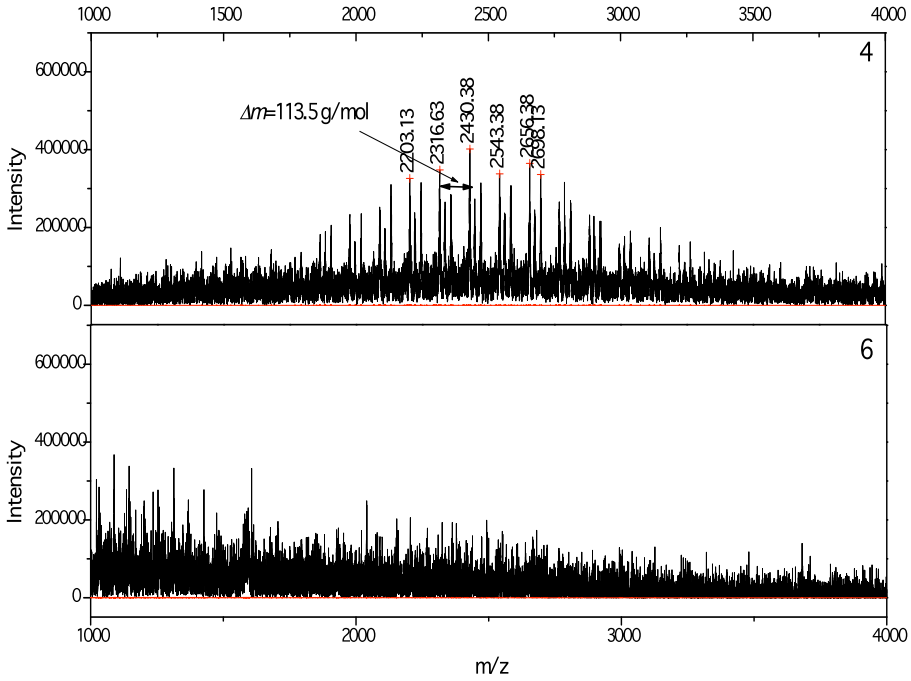


Figure S7. ESI-MS spectra of PNiPAAM (4) before click reaction with 2 (top) and concentrated crude mixture after CuAAC reaction and copper removal (6, bottom).

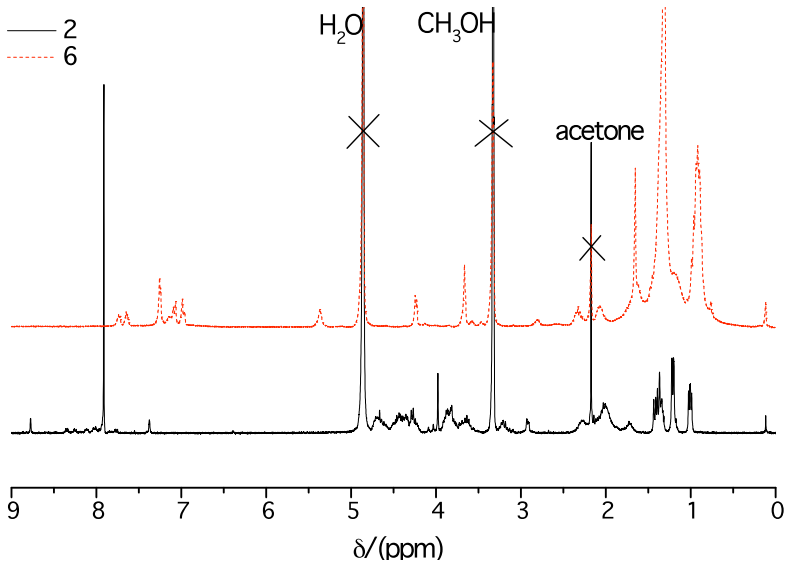


Figure S8. ^{1}H NMR spectra of purified eicosapeptide 2 (bottom) and chimera 6 obtained after the CuAAC reaction.

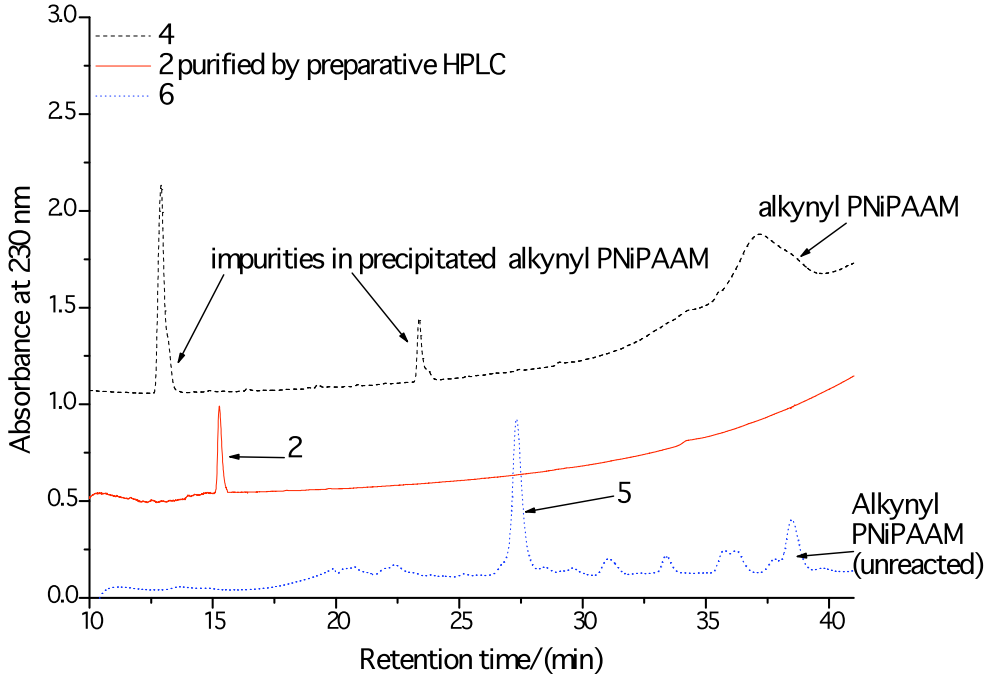


Figure S9. HPLC chromatograms of purified eicopeptide 2, PNiPAAM and chimera 6 obtained after the CuAAC reaction. The gradient used was 0-100% B over 40 minutes.

Thiol functionalisation of polymer peptide chimeras 5 and 6 (General procedure). To a solution of chimera **6** (4 mg, 0.90 mmol) dissolved in methanol (3 mL) was added sodium borohydride in methanol (2% w/v, 1 mL). The reaction was stirred for 2 hours after which the pH was adjusted to neutral using acetic acid. The product was purified by dialysis against water for 3 days and the solution was analysed by UV-vis before lyophilisation.

NB: a peak with maximum at 288 nm was observed in the UV-vis spectrum. This was found to be due to traces of a CuBr/PMDETA complex which had coeluted during silica chromatography.

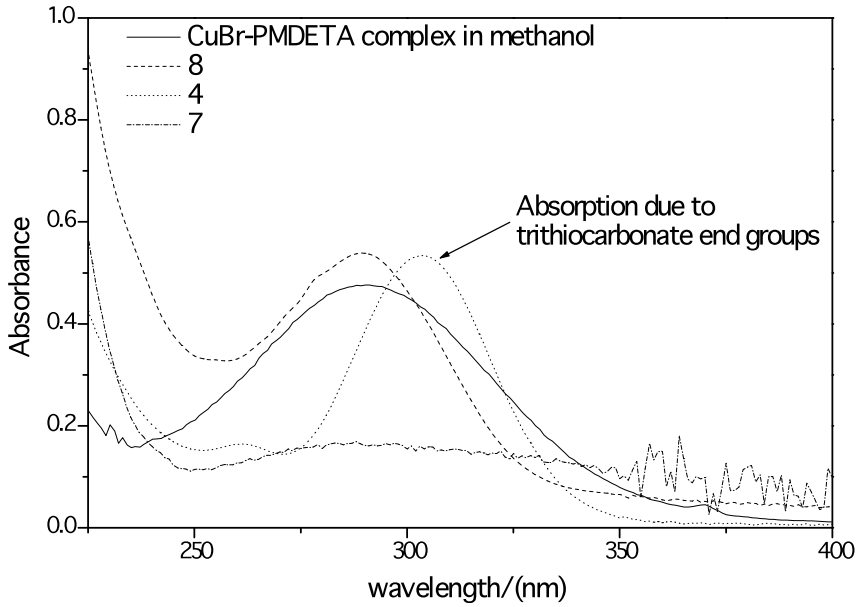


Figure S10. UV spectra of pure non-reduced PNiPAAM polymer (with a trithiocarbonate end group), CuBr-PMDETA and chimeras 7 and 8 (after reduction with sodium borohydride) in methanol.

Typical functionalisation of chimera with pyrene tag – Chimera 8. In an aluminium foil covered vial, freeze dried chimera **8** (4.0 mg, 0.0009 mmol) was dissolved in DMF (3.0 ml) and the vessel was sparged with nitrogen for one hour. An excess of TCEP (25.7 mg, 0.0897 mmol) was dissolved by sonication in deoxygenated DMF (1.5 ml) and the vial was also flushed with nitrogen for one hour. The degassed TCEP solution was carefully transferred to the vial containing **8** and the resulting solution was stirred for 5 h under nitrogen. A previously degassed solution of N-1 pyrene maleimide (40 mg, 0.0135 mmol) in DMF 1.5 ml, was then added followed by 5μL of ethylenediamine and the reaction was stirred at room temperature for 16 h under a slow flow of nitrogen. The reaction mixture was then diluted with water and transferred into a dialysis bag for dialysis against 10 L of deionised water for 8 hours. The sample was then filtered through a membrane filter (0.45 micron) to remove precipitated pyrene maleimide. The resulting aqueous polymer solution was dialysed for a further 16 h and refiltered before being left for further dialysis against water for 5 days. An aliquot of the sample was degassed with nitrogen for 15 minutes and the fluorescence emission spectrum of the solution was then obtained with the excitation wavelength set at 340 nm and a slit width of 5nm. To estimate the yield of

the reaction, the sample solution was then freeze-dried and dissolved in methanol followed by measurement of the absorbance at 338 nm (extinction coefficient of pyrene maleimide at 338 nm in methanol is 40,000 $cm^{-1}M^{-1}$).[5] An approximate conjugation yield to obtain **10** = 33% (yield for **9** = 34%).

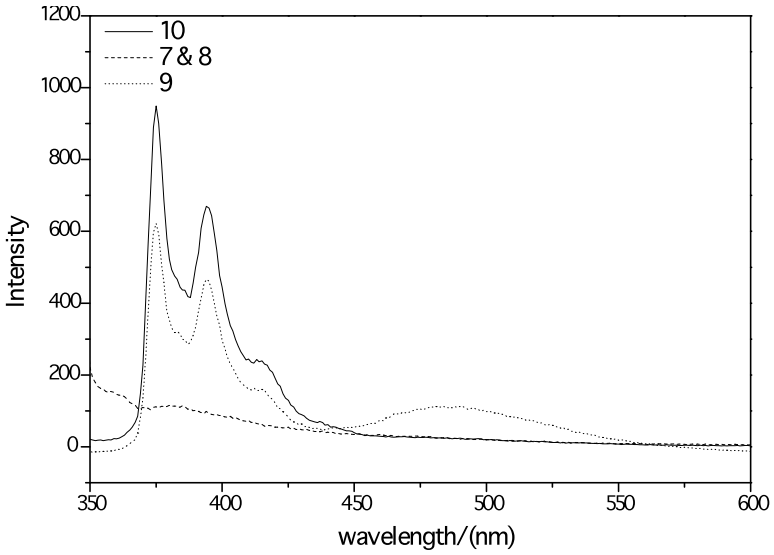


Figure S11. Fluorescence emission spectra of aqueous solutions of chimera 9 and 10 before and after conjugation with pyrene maleimide.

Determination of LCST of PNiPAAM by DSC. A PNiPAAM polymer solution of 5 mg/mL (M_n(NMR) =2600 g/mol, PDI=1.2) was made by sonicating the polymer in cold water and 20 mg of this solution was weighed into a DSC sample holder for analysis. The sample was initially cooled to 4 ^oC and held constant for 10 minutes before the heating cycle was started. The heating and cooling rate was 1 ^oC/min up to 80 ^oC and down to 4 ^oC. The results reported were obtained from the second run with the LCST being taken as the lowest point in the trough of the endotherm corresponding to the LCST transition.

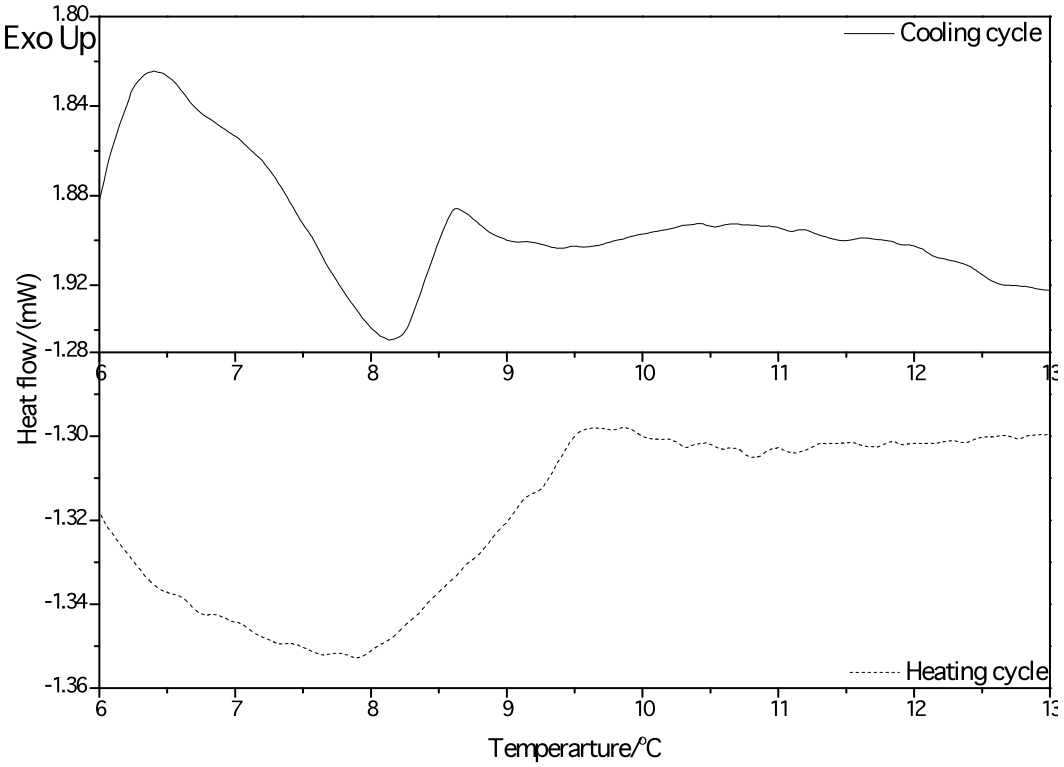


Figure S12. DSC thermograms of 4 in water showing the transition that takes place during heating and cooling cycles.

Self assembly. Polymer-peptide chimeras **5** and **6** were dissolved in methanol (9mg/mL) and the solutions were slowly diluted with water (0.04 mL/min, 25 ^{o}C) until the concentration of the solutions were 1mg/ml. Each solution was filtered through membrane filters (0.45 μm) and half of each of the solutions was transferred into separate vials which were placed in an oil bath maintained at 50 ^{o}C. The solutions were stirred at 50 ^{o}C for 12 days before analysis by DLS and TEM.

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