

Supporting Information

Tunable thermo-responsive polymer-protein conjugates *via* a combination of  
nucleophilic thiol-ene “click” and SET-LRP

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Reagents

Salmon calcitonin acetate (sCT) was purchased from PolyPeptide Laboratories (Hillerod, Denmark) and stored at 4°C. Tris(2-carboxyethyl) phosphine (TCEP), 2-hydroxyethyl acrylate and 2-bromoisobutyrate, diethylene glycol methyl ether methacrylate (DEGMEMA) were purchased from Sigma-Aldrich and stored at 4°C. Wang Resin (3 mmol g<sup>-1</sup>) was obtained from Avescia. Copper wire (diameter 0.25 mm) was cleaned with hydrochloric acid, washed with water and dried prior to use.

RP-HPLC was carried out using a Varian PLRP-S 100Å μm 250 × 4.6 mm column. The HPLC system comprised of two Gilson 306 pumps a Gilson 811B mixer and a Gilson 805 manometric module; the sample was injected using a SPARK Endurance autosampler. Sample detection was carried out using two UV detectors connected in series, a Jasco-975 and Knauer K-2001 monitored at λ = 280 nm respectively. The mobile phases used were: a) Mobile phase A: 90 % v/v water, 10 % v/v MeCN (far UV) with 0.05 % v/v TFA; b) Mobile phase B: 100 % v/v MeCN (far UV) with 0.04 % v/v TFA. The column was equilibrated for 10 minutes by washing with mobile phase A before sample injection. To ensure that the column was thoroughly washed before each sample injection and to prevent the build up of contaminants, the gradient included a final washing step whereby the concentration of mobile phase B was

increased to 80 %. HPLC grade solvents/reagents were used in all experiments. The gradient used is given in Table 1.

| <u>Time (min)</u> | <u>% Mobile phase A</u> | <u>% Mobile phase B</u> |
|-------------------|-------------------------|-------------------------|
| 0                 | 90                      | 10                      |
| 27                | 40                      | 60                      |
| 37                | 20                      | 80                      |
| 48                | 20                      | 80                      |
| 52                | 90                      | 10                      |
| 60                | 90                      | 10                      |

**Table 1.** Gradient used for RP-HPLC analysis of the conjugates studied in this work.

#### MALDI-ToF Analysis

Mass spectra were acquired by MALDI-ToF (matrix-assisted laser desorption ionisation time-of-flight) mass spectrometry using a Bruker Daltonics Ultraflex II MALDI-ToF mass spectrometer, equipped with a nitrogen laser delivering 2 ns laser pulses at 337 nm with positive ion ToF detection performed using an accelerating voltage of 25 kV. Samples were prepared by layering 2,5-dihydroxybenzoic acid (DHB) (2  $\mu\text{L}$  of a 10 mg  $\text{mL}^{-1}$  solution), followed by 2  $\mu\text{L}$  of a trifluoroacetic acid (10 mg  $\text{mL}^{-1}$  solution) and the analyte solution (2  $\mu\text{L}$  of a 10 mg  $\text{mL}^{-1}$  solution).

#### Gel Permeation Chromatography (GPC) Analysis

Offline GPC analysis performed on a system set at 50°C fitted with 2  $\times$  PL Mixed D (300  $\times$  7.5 mm) columns and a 5  $\mu\text{m}$  guard column with dimethylformamide as the mobile phase. The GPC system was equipped with ultraviolet (UV) (set at 280 nm) and differential refractive index (DRI) detectors which was calibrated with linear narrow molecular weight PMMA standards ranging from a dimer (200 Da) up to 772,000 Da using a second order polynomial fit.

### LCST measurements

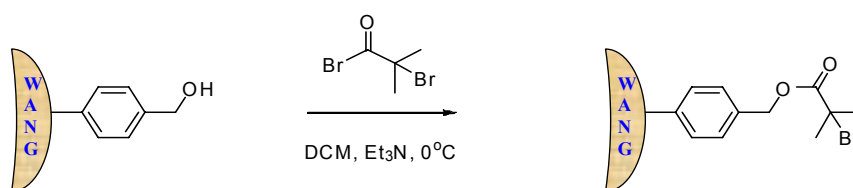
Cloud-point analysis was performed on an OptiMelt MPA100 system by Stanford Research Systems. Conjugates were dissolved in 18 M $\Omega$  water at a concentration of 1 mg mL<sup>-1</sup> and heated at a rate of 0.5 °C min<sup>-1</sup>. Two runs were performed simultaneously and an average of both runs plotted to determine the cloud point of the respective conjugate.

### DLS analysis

Dynamic light scattering was conducted using a Nano-Zs from Malvern Instruments, UK. Scattered light was detected at 173 ° and hydrodynamic radii determined using the manufacturer's software (using Cumulants analysis). Measurements were conducted using the solvent indicated in the text. Hydrodynamic radii were determined from an average of 15 experiments, and the number-weighted average result displayed.

### Experimental Procedures

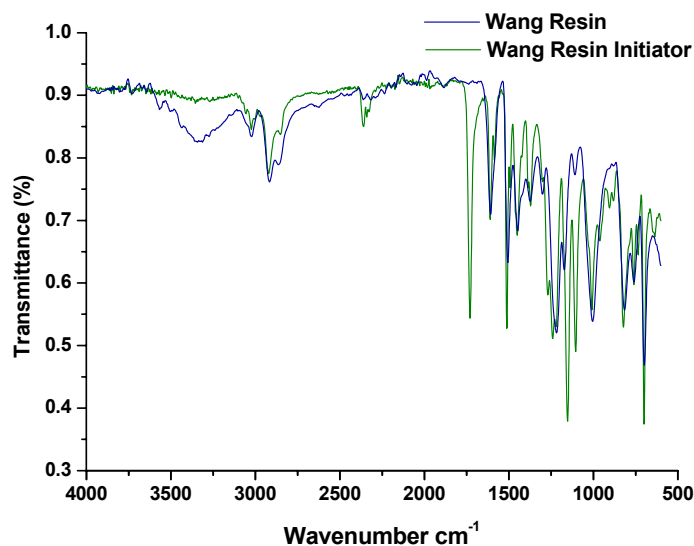
#### Synthesis of Initiator-Functional Wang Resin



10.00g of Wang resin (3 mmolg<sup>-1</sup> loading) was suspended in dry dichloromethane (100mL) and cooled to 0°C in an ice bath. Triethylamine (21.1 mL, 150 mmol) was added to the solution and left to stir gently for 5 minutes. 2-bromoisobutyryl bromide (18.5 mL, 150 mmol) was added dropwise to the Wang suspension over a period of 30 minutes and left overnight at room temperature overnight following complete addition. The suspension was then filtered and washed with copious amounts of dichloromethane, tetrahydrofuran, acetone, methanol, water followed by further

washings with methanol, tetrahydrofuran and dichloromethane. The beads were isolated and dried under vacuum overnight to yield the modified beads (10.04 g).

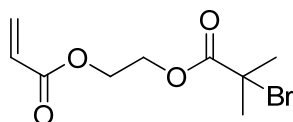
IR spectroscopy confirmed the presence of ester moiety of the initiator fragment



( $\nu_{\text{COOR}} = 1732 \text{ cm}^{-1}$ ).

Figure S1. Infrared spectrum of initiator-functional Wang resin.

#### Acryloyloxyethyl 2-Bromoisobutyrate



2-hydroxyethyl acrylate (5.0 g, 43.0 mmol) was dissolved in 200 mL of dry THF and cooled to 0°C in an ice bath. Triethylamine (6.29 mL, 47.3 mmol) was added to the solution and left to stir for 10 minutes. 2-bromoisobutyryl bromide (5.85 mL, 47.3 mmol) was dissolved in 30 mL of THF and added dropwise to the stirring solution over a period of 20 minutes, whereby an immediate white precipitate was observed. Following complete addition, the solution was allowed to warm to room temperature and left to stir for 12 hours. The solution was then filtered to remove salts and the solvent removed under reduced pressure. The crude mixture was redissolved in 150 mL of DCM and washed with water (2 × 100 mL), saturated NaHCO<sub>3</sub> (2 × 100 mL),

water (100 mL) and brine (100 mL). The organic layer was dried with anhydrous magnesium sulfate, filtered and the solvent removed under reduced pressure. The oil was then purified by flash chromatography (CC, SiO<sub>2</sub>) (100% DCM) to yield the product as a colourless oil (10.68 g, 93.6% yield).

<sup>1</sup>H NMR (CDCl<sub>3</sub>): 1.92 (s, 6H), 4.41 (s, 4H), 5.85 (d, *J* = 7.3 Hz, 1H), 6.12 (m, 1H), 6.42 (d, *J* = 7.3 Hz, 1H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 30.79, 55.48, 61.87, 63.62, 128.07, 131.59, 165.91, 171.60. IR (neat, cm<sup>-1</sup>) ν = 1732, 1263, 1190, 1159. HRMS (ES+) calcd for C<sub>9</sub>H<sub>13</sub>BrO<sub>4</sub> [M + Na]<sup>+</sup> 286.9889, observed 286.9887.

#### Triethylene glycol methyl ether methacrylate (TEGMEMA)

Triethylene glycol monomethyl ether (20.00 g, 0.12 mol) was dissolved in 250 mL of dry THF and cooled to 0°C in an ice bath. Triethylamine (18.67 mL, 0.13 mol) was added to the solution and left to stir for 10 minutes. Methacryloyl chloride (12.96 mL, 0.13 mol) was added dropwise to the solution over a period of 30 minutes and the solution allowed to warm to room temperature. After 12 hours, the white precipitate was filtered and the solvent removed. The crude mixture was dissolved in 150 mL of dichloromethane and washed with water (2 × 100 mL), saturated NaHCO<sub>3</sub> (2 × 100 mL), water (100 mL) and brine (100 mL). The organic layer was dried over MgSO<sub>4</sub>, filtered and the solvent removed under vacuum. The obtained oil was purified by flash chromatography (CC, SiO<sub>2</sub>) (100% petroleum ether followed by 8:2 petroleum ether : diethyl ether and finally 1:1 petroleum ether : diethyl ether) to elute the product as a colourless oil (23.93 g, 84.6% yield).

<sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.92 (s, 3H, CH<sub>3</sub>), 3.35 (s, 3H, OCH<sub>3</sub>), 3.52 (t, *J* = 5.0 Hz, 2H), 3.69 (m, 6H), 3.72 (t, *J* = 5.0 Hz, 2H), 4.27 (t, *J* = 4.8 Hz, 2H), 5.54 (s, 1H), 6.10 (s, 1H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 18.38, 59.11, 63.96, 69.4, 70.68, 70.73, 70.74, 72.02, 125.78, 136.26, 167.43. IR (neat, cm<sup>-1</sup>) ν = 3000-2700, 1719, 1639, 1454, 1321, 1296, 1170. HRMS (ES+) calcd for C<sub>11</sub>H<sub>20</sub>O<sub>5</sub><sup>+</sup> [M + Na]<sup>+</sup> 255.1203, observed 255.1207.

#### Synthesis of sCT-macroinitiator

Salmon Calcitonin (sCT) (30 mg, 8.7 mmol) was dissolved in 3.0 mL of deionised water along with tris(2-carboxyethyl)phosphine (TCEP) (4.3 mg, 14.8 mmol). The solution was left to stir for 30 minutes and sampled to confirm quantitative disulfide reduction. 2.0 mL of phosphate buffer (pH 6.5, 500 mM) was added to the solution, along with 'inomer' (16.2 mg, 61.2 mmol) in 1.0 mL of DMSO. The solution was left to stir for 1 hour and sampled for RP-HPLC analysis. RP-HPLC confirmed the appearance of a new peak, with no remaining reduced sCT present. MALDI-TOF analysis of the new peak confirmed the new peak as the expected product. The solution was diluted with 30 mL of a 50:50 water:methanol solution and dialysed (MWCO 1kDa) against the same solution for 2 days, followed by dialysis against water for 1 day. Lyophilisation of the solution yielded the product as a white powder (25.8 mg) in a good yield of 74.5%.

MALDI-ToF analysis of the obtained di-functional sCT can be seen in Figure 2 in the main article.

### Polymerisation Studies

#### Solution-Based Initiator

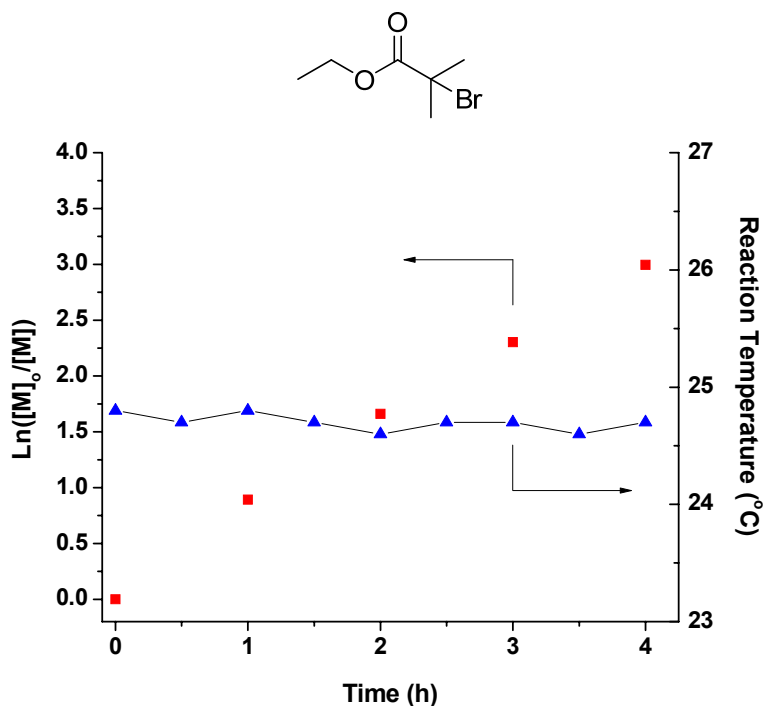


Figure S2. Kinetic plot and temperature profile of DEGMEMA polymerisation by SET-LRP using solution-based initiator (ethyl-2-bromo isobutyrate). Reaction temperature was monitored using an *in situ* thermocouple.

Resin-based Initiator

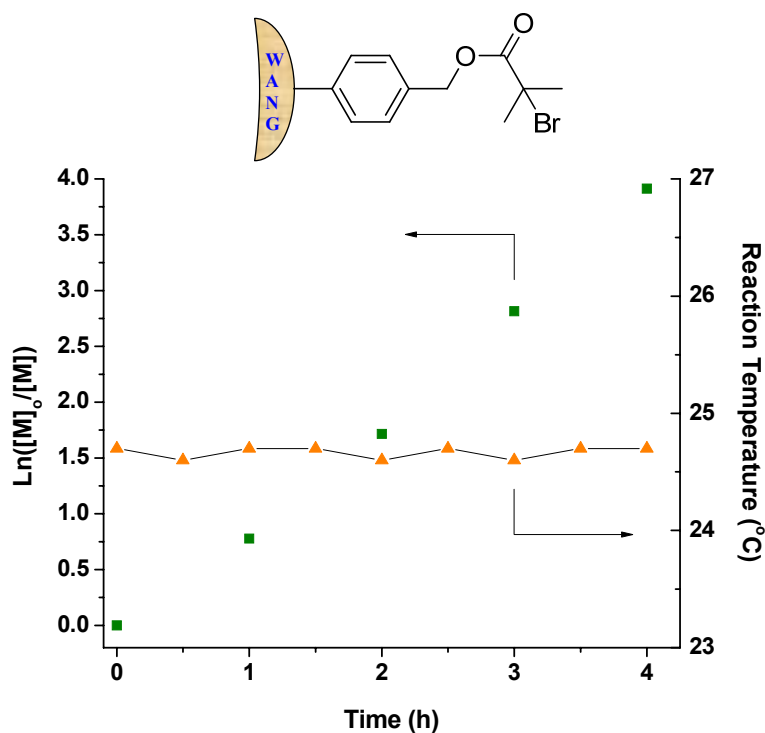


Figure S3. Kinetic plot and temperature profile of DEGMEMA polymerisation by SET-LRP using solid-supported initiator. Reaction temperature was monitored using an *in situ* thermocouple.

### sCT-poly(DEGMEMA) Conjugate

The synthesised salmon calcitonin macroinitiator (4.2 mg, 1.3  $\mu\text{m}$ ) was added to a schlenk tube along with the initiator-functional Wang resin (0.442 g, 1.32 mmol), 5 cm of copper (0) wire (0.25 mm diameter), copper (II) bromide (29.6 mg, 0.13 mmol) and a magnetic stirrer bar. The schlenk was sealed and thoroughly degassed and purged with nitrogen. In a separate schlenk tube, di(ethylene glycol) methacrylate (5.00 g, 26.5 mmol), dimethyl sulfoxide (10 mL), *N,N,N',N',N''*-pentamethyldiethylene triamine (0.43 mL, 1.99 mmol) and mesitylene (0.37 mL, 2.6 mmol) were added and subjected to four freeze-pump thaw cycles. The degassed solution was transferred *via* cannula to the first schlenk tube and immersed in an oil bath at 25 °C for 3 h at which point the polymerisation was quenched in liquid nitrogen and filtered to remove solids. The remaining solution was dialysed against a 50:50 mixture of water:methanol for 3 days and 1 day against pure water. The solution was then lyophilised and isolated as a colourless oil.

GPC analysis of the sCT-poly(DEGMEMA) conjugate showed a well-defined peak corresponding to the conjugate, as well as some aggregation of the product during analysis.



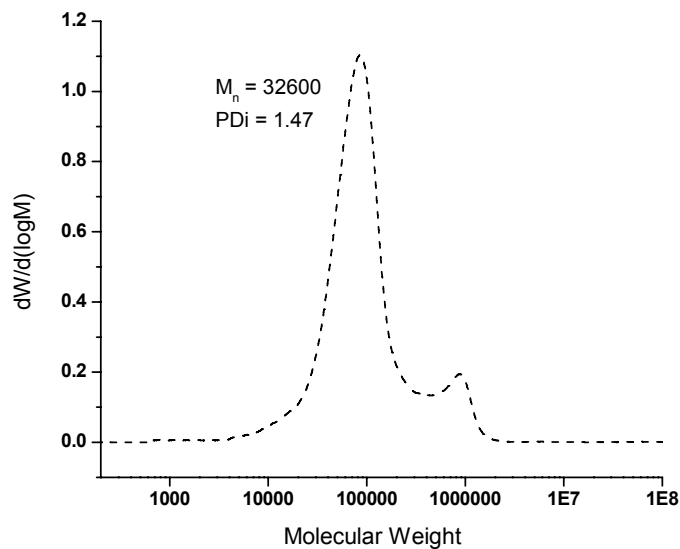


Figure S4. DMF-GPC analysis of sCT-poly(DEGMEMA) conjugate ( $M_n$  32600, PDI 1.47). A peak at higher molecular weight is observed, corresponding to aggregation of the conjugate during GPC analysis.

The conjugate was analysed by GPC using a UV detector set at 280 nm in order to detect the signal corresponding to sCT. A UV signal corresponding to sCT was observed in both the major conjugate peak, as well as the aggregated conjugate, showing that all macromolecular species originated from the polypeptide macroinitiator.

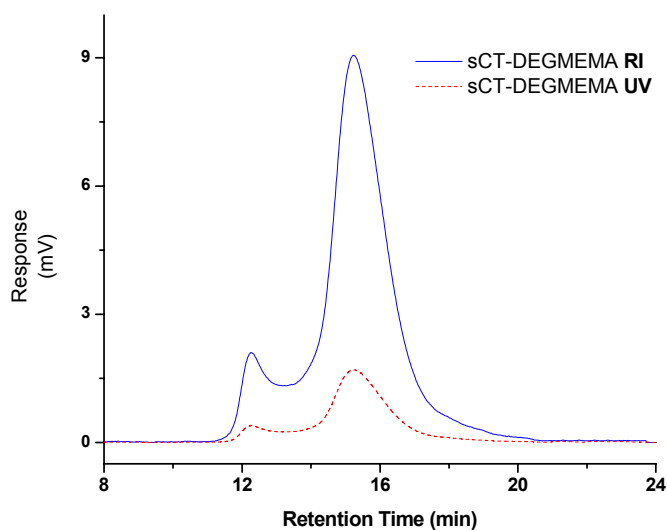


Figure S5. GPC analysis of the sCT-poly(DEGMEMA) conjugate by RI and UV.

The extent of aggregation observed by GPC, the sCT-poly(DEGMEMA) conjugate was analysed by DLS. The conjugate was dissolved in DMF at a concentration of 1 mg ml<sup>-1</sup>. Aggregation was shown to be negligible, with the only significant signal corresponding to the small conjugate aggregates, at around 6.2 nm.

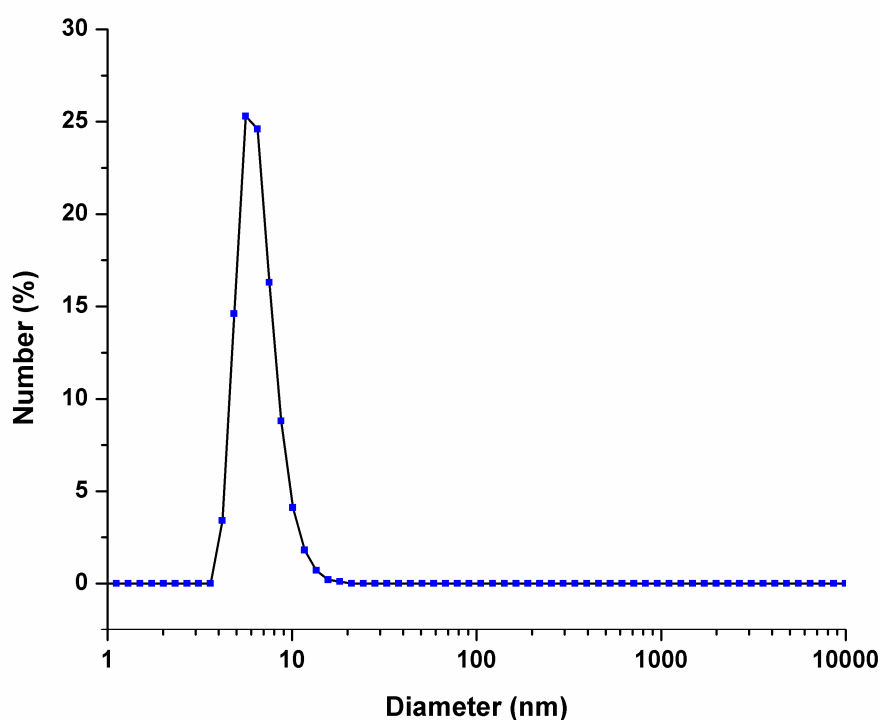


Figure S6. DLS analysis of the sCT-poly(DEGMEMA) conjugate in DMF with largely unimolecular species of around 6.2 nm observed..

### Cloud-Point Analysis

A solution of the sCT-poly(DEGMEMA) conjugate, at a concentration of 1 mg ml<sup>-1</sup> was heated from 15°C to 35°C at a rate of 0.5 °C min<sup>-1</sup>. Two samples were run

simultaneously and an average of both runs plotted and used to determine the cloud-point.

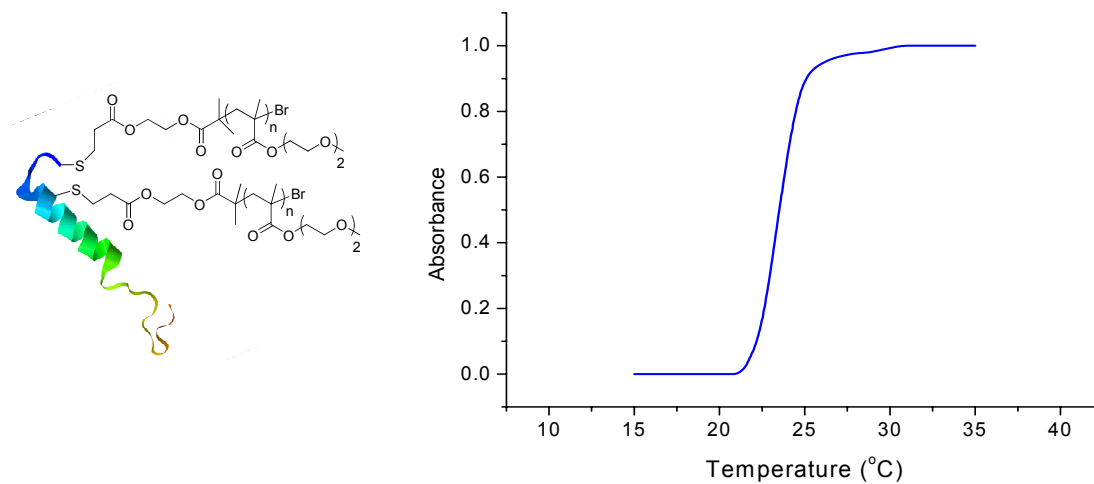


Figure S7. Cloud-point analysis of sCT-poly(DEGMEMA) conjugate (24°C).

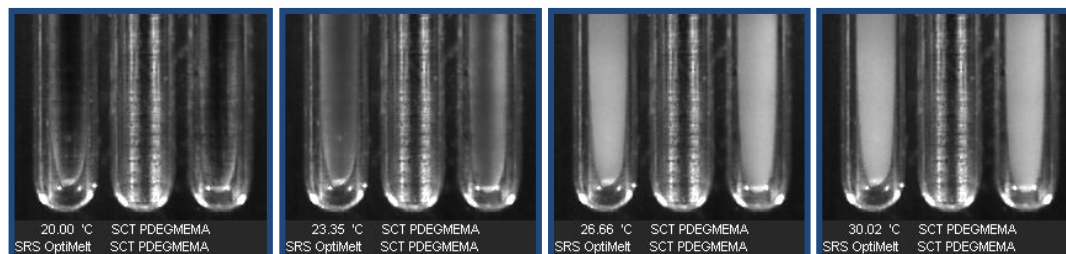


Figure S8. Visual representation of sCT-poly(DEGMEMA) conjugate aggregation.

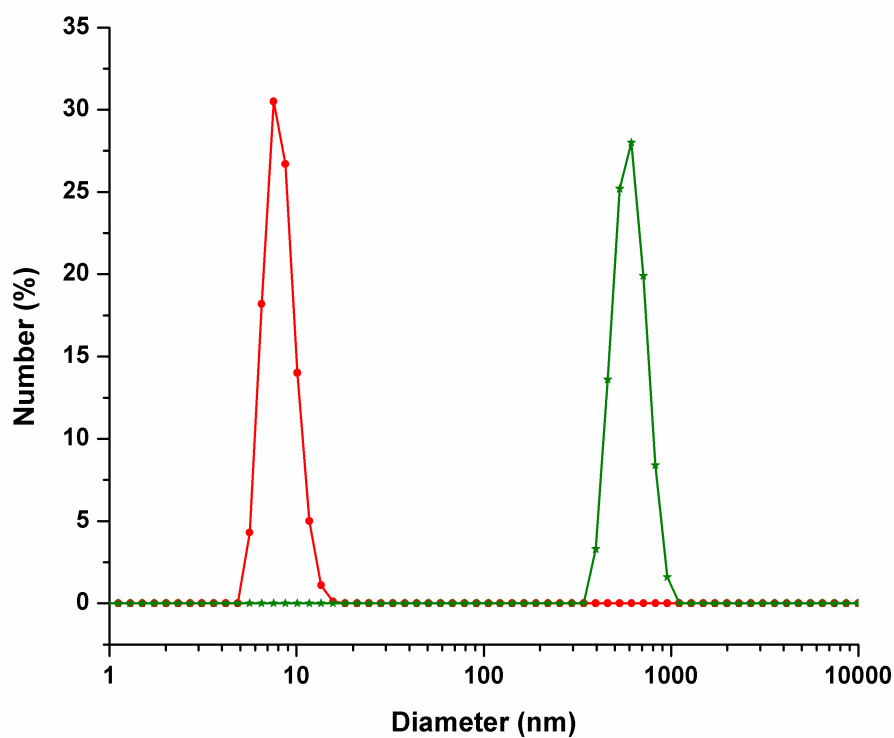


Figure S9. DLS analysis of sCT-poly(DEGMEMA) conjugate at 23°C (red trace) and 26°C (green trace).

DLS was used to determine the size distributions of conjugates upon heating above their LCST. At 23°C, small aggregates (7.2 nm) exist and upon heating above its LCST at 26°C large aggregates of around 620 nm are observed.

sCT-poly(TEGMEMA) Conjugate

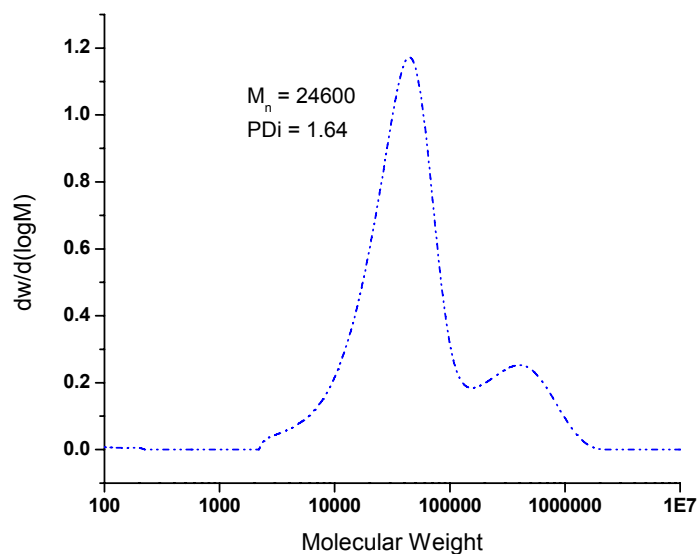


Figure S10. DMF-GPC analysis of sCT-poly(TEGMEMA) conjugate ( $M_n$  24600, PDI 1.64). A peak at higher molecular weight is observed, corresponding to aggregation of the conjugate during GPC analysis.

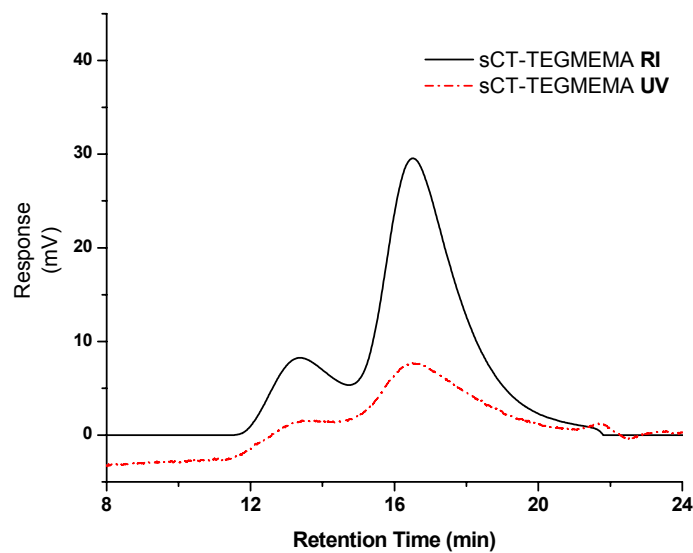


Figure S11. GPC analysis of the sCT-poly(TEGMEMA) conjugate by RI and UV (280 nm).

The extent of aggregation observed by GPC, the sCT-poly(DEGMEMA) conjugate was analysed by DLS. The conjugate was dissolved in DMF at a concentration of 1 mg ml<sup>-1</sup>. The extent of aggregation was shown to be negligible, with the only significant signal corresponding to small aggregates at around 7.4 nm.

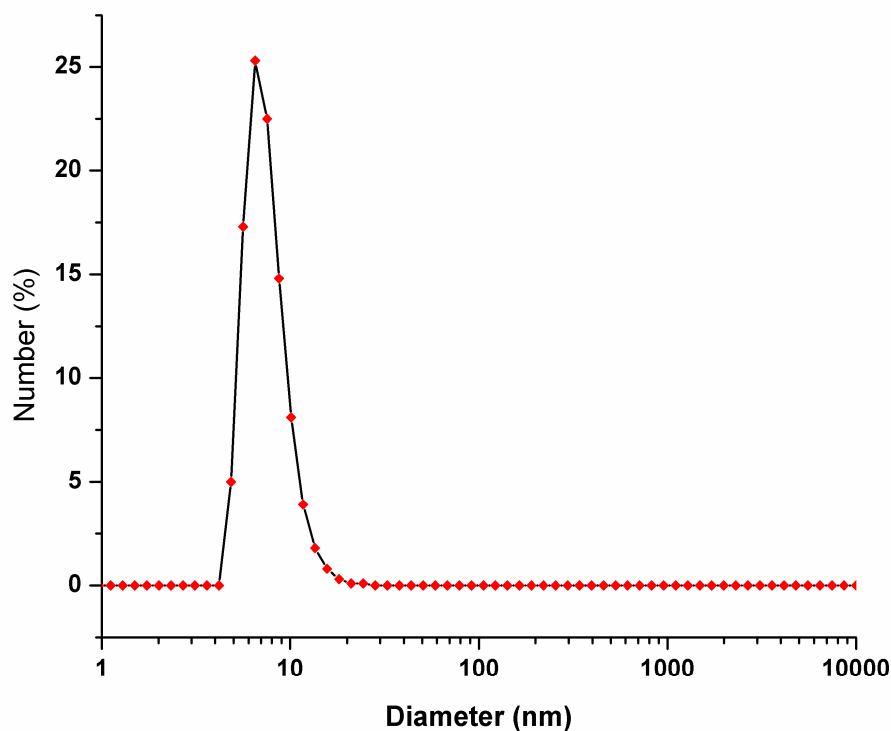


Figure S12. DLS analysis of the sCT-poly(TEGMEMA) conjugate in DMF with largely unimolecular species of around 7.4 nm observed.

A solution of the sCT-poly(TEGMEMA) conjugate, at a concentration of 1 mg ml<sup>-1</sup> was heated from 40°C to 65°C at a rate of 0.5 °C min<sup>-1</sup>. Two samples were run simultaneously and an average of both runs plotted and used to determine the cloud-point.

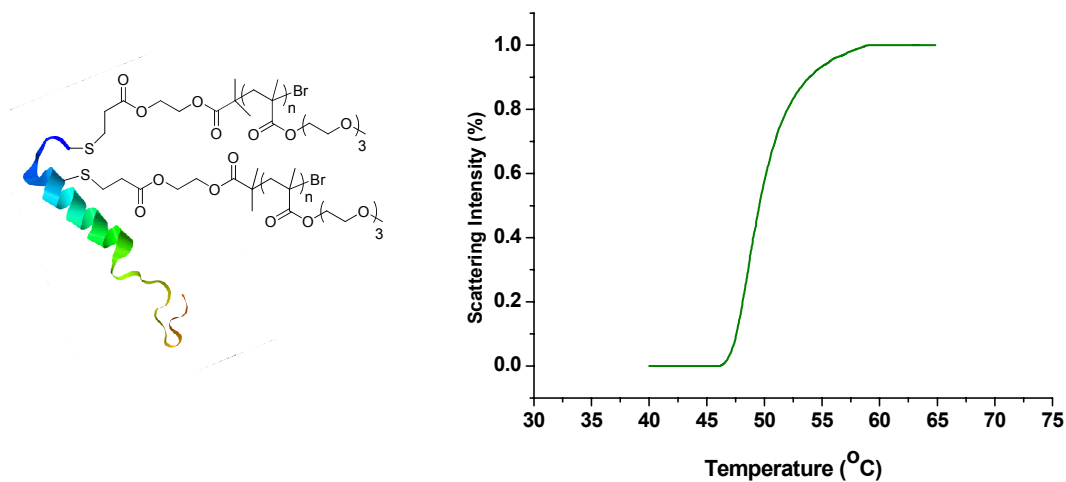


Figure S13. Cloud-point analysis of sCT-poly(TEGMEMA) conjugate.

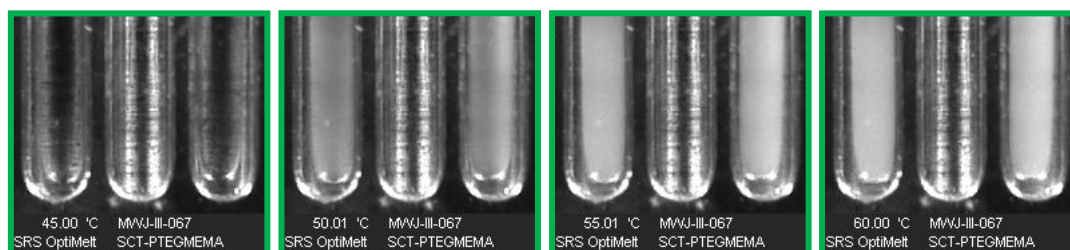


Figure S14. Visual representation of sCT-poly(TEGMEMA) conjugate aggregation.

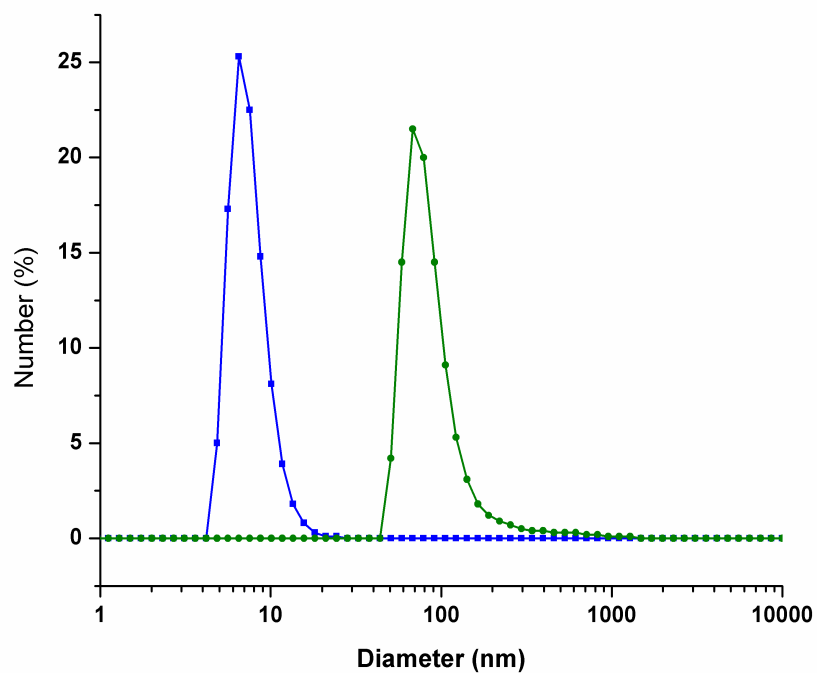


Figure S15. DLS analysis of sCT-poly(TEGMEMA) conjugate at 48°C (blue trace) and 52°C (green trace).

DLS was used to determine the size distributions of conjugates upon heating above their LCST. At 48°C, small aggregates (7.2 nm) exist and upon heating above its LCST at 52°C large aggregates of around 78 nm are observed.



sCT-poly(DEGMEMA-co-TEGMEMA) Conjugate

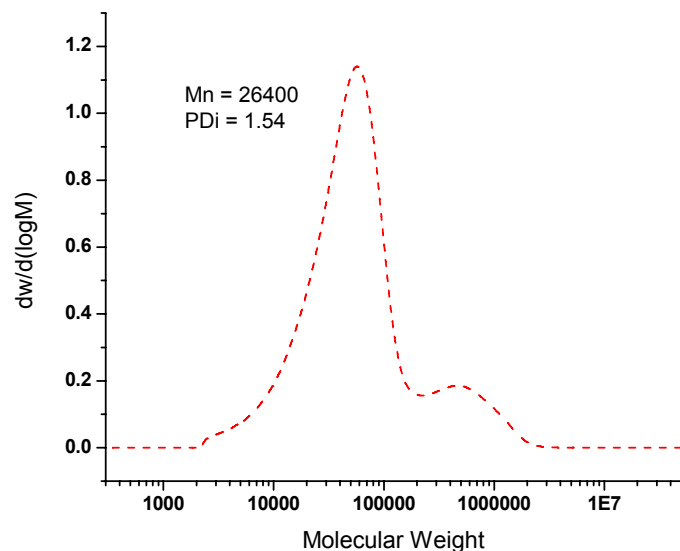


Figure S16. DMF-GPC analysis of sCT-poly(DEGMEMA-co-TEGMEMA) conjugate (Mn 26400, PDi 1.54). A peak at higher molecular weight is observed, corresponding to aggregation of the conjugate during GCP analysis.

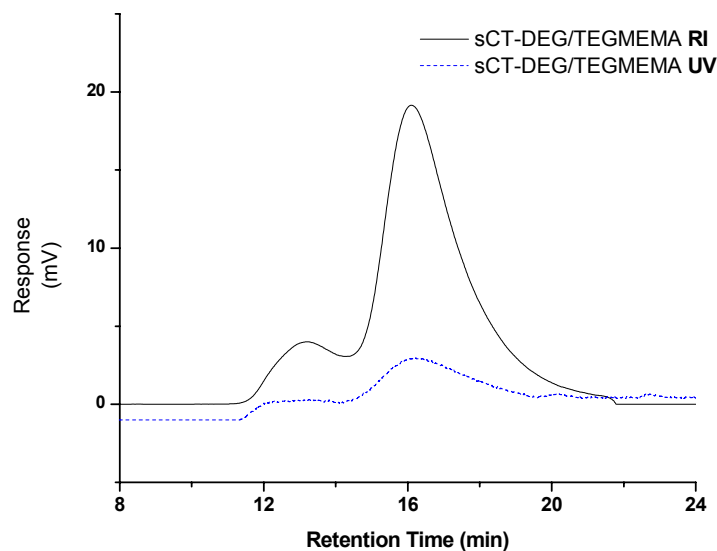


Figure S17. GPC analysis of the sCT-poly(DEGMEMA-co-TEGMEMA) conjugate by RI and UV (280 nm).

The extent of aggregation observed by GPC, the sCT- poly(DEGMEMA-co-TEGMEMA) conjugate was analysed by DLS. The conjugate was dissolved in DMF

at a concentration of  $1 \text{ mg ml}^{-1}$ . Aggregation was shown to be negligible, with only small aggregates of around 7.4 nm observed.

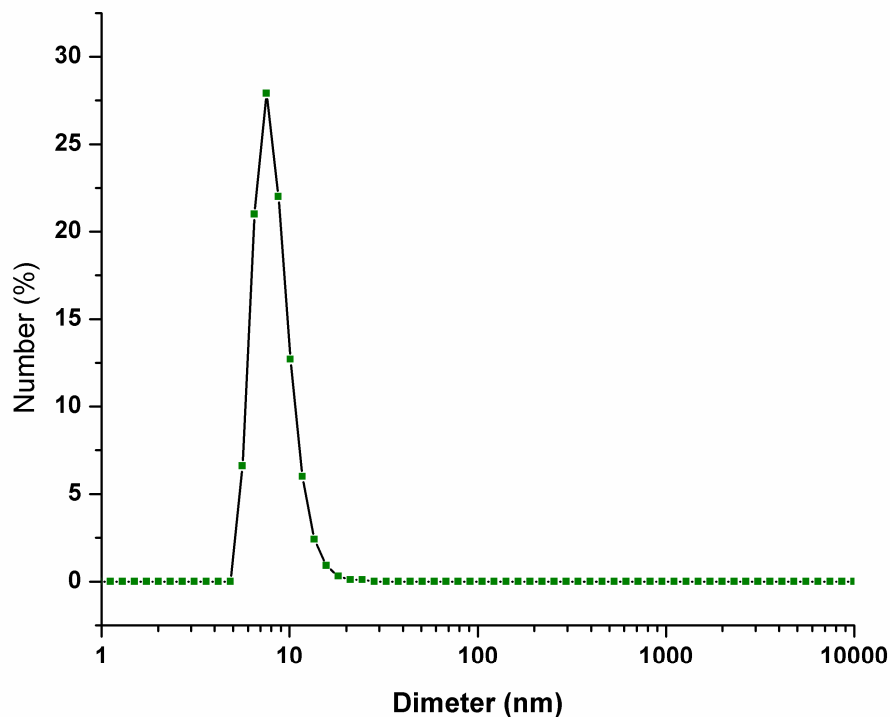


Figure S18. DLS analysis of the sCT-poly(DEGMEMA-*co*-TEGMEMA) conjugate in DMF with largely unimolecular species of around 7.4 nm observed.

A solution of the sCT-poly(DEGMEMA-*co*-TEGMEMA) conjugate, at a concentration of  $1 \text{ mg ml}^{-1}$  was heated from  $25^\circ\text{C}$  to  $55^\circ\text{C}$  at a rate of  $0.5^\circ\text{C min}^{-1}$ . Two samples were run simultaneously and an average of both runs plotted and used to determine the cloud-point.

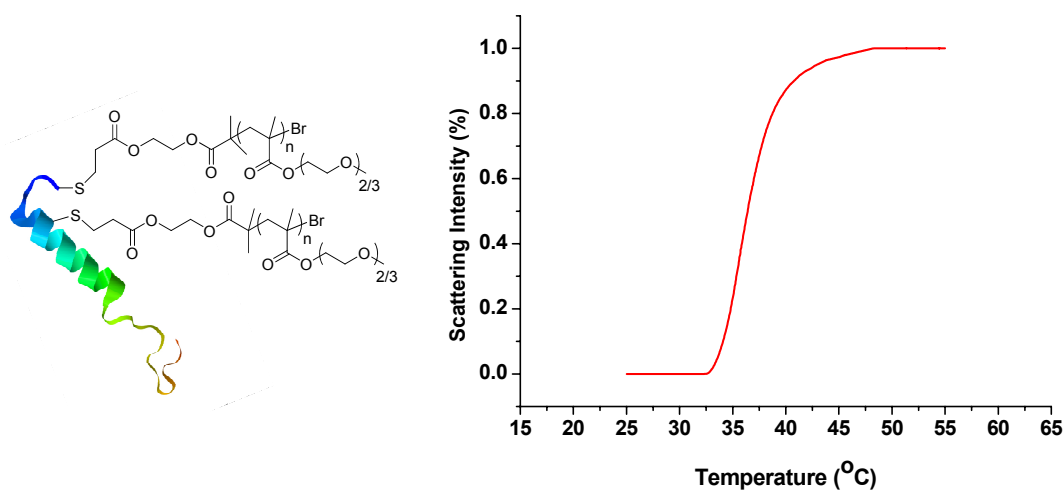


Figure S19. Cloud-point analysis of sCT-poly(DEGMEMA-*co*-TEGMEMA) conjugate.

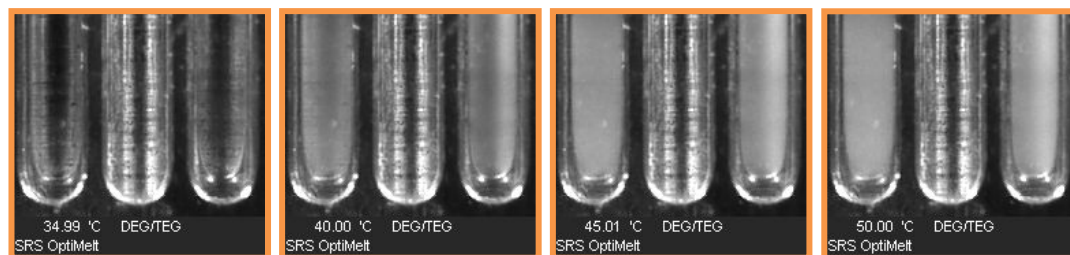


Figure S20. Visual representation of sCT-poly(DEGMEMA-*co*-TEGMEMA) conjugate aggregation.

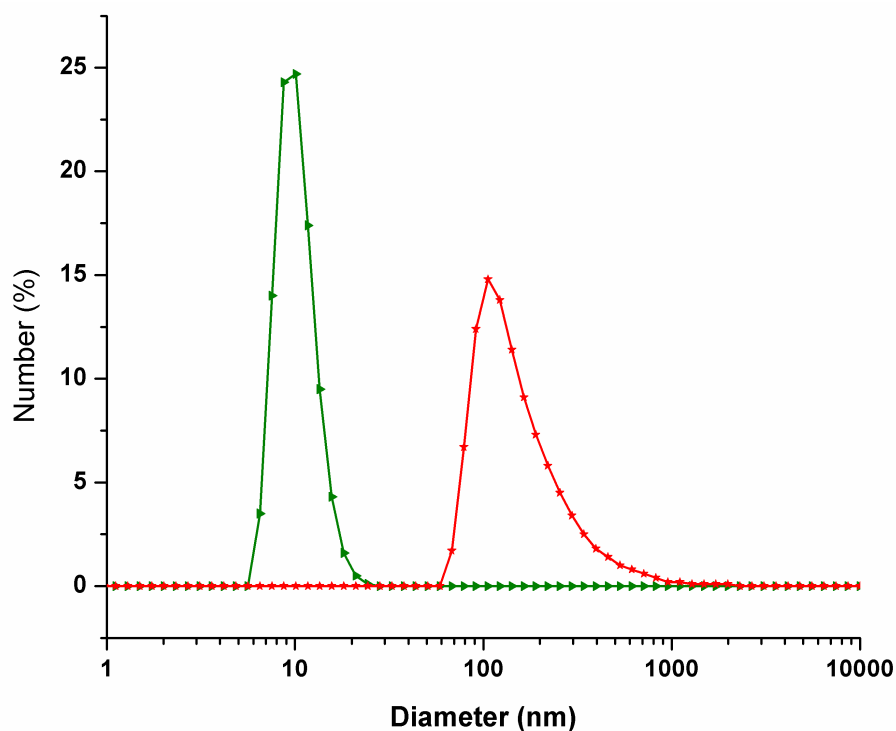


Figure S21. DLS analysis of sCT-poly(DEGMEMA-co-TEGMEMA) conjugate at 35°C (red trace) and 39°C (green trace).

DLS was used to determine the size distributions of conjugates upon heating above their LCST. At 35°C, only small aggregates (9.2 nm) are observed and upon heating above its LCST at 39°C larger aggregates of around 105 nm are formed.