Facile synthesis of responsive nanoparticles with reversible, tuneable and rapid thermal transitions from biocompatible constituents

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Materials

Tetraethyleneglycol (TEG) (Aldrich, 99%), triethylamine (TEA) (Sigma Aldrich, \geq 99.5%), 2bromoisobutyryl bromide (Aldrich, 98%), lactide (Aldrich), glycolide (Sigma, >99%), poly(ethylene glycol) methyl ether methacrylate (PEGMEMA) (Average M_n *ca.* 475, Aldrich), poly(propylene glycol)methacrylate (PPGMA) (Average M_n *ca.* 375, Aldrich), copper(II) bromide (Alfa Aesar, 99%), *N*,*N*,*N*'',*N*''-pentamethyldiethylenetriamine (PMDTA) (Aldrich, 99%), tin(II) 2-ethylhexanoate (Sn (EH)₂) (Aldrich, 95%), 3-chloropropyltrimethoxysilane (Aldrich, 97%), Rhodamine 6G (Fluka), 2-butanone (Alfa Aesar, 99%), CDCl₃ (Aldrich, 99.8% D), anhydrous THF (Aldrich, 99.8%) were used as received.

Acetone, chloroform, hexane and diethyl ether (HPLC grade) were purchased from Fisher Scientific. Deionized water (Maxima HPLC grade Elga) was used during preparation of nanoparticles. Dialysis membrane (molecular weight cut-off *ca.* 3,500) was obtained from Spectrum Laboratories Inc. Silica gel used for column chromatography was obtained from Acros Organics.

Analysis

Number average molecular weight (M_n), weight average molecular weight (M_w) and polydispersity Index (PDI = M_w/M_n) of the prepared copolymers were obtained by Gel Permeation Chromatography (GPC) (PL-120 and PL-50 Polymer Labs) with a refractive index detector. The columns (7.5 x 300 mm Resipore Mixed-D, 2 in series) were eluted by chloroform and calibrated with polystyrene standards. All calibration and analysis were performed at 40 °C and a flow rate of 1 mL min⁻¹. All of the products were dissolved in chloroform and passed through 0.2 µm filter prior to column chromatography.

¹H NMR spectra were recorded at 20 °C on a Bruker-DRX instrument operating at 400 MHz. Chemical shifts (δ) are reported in parts per million (ppm), referenced to CDCl₃ (δ 7.26). The data was processed using the TOPSPIN 2 software.

Infrared spectra were measured on an Avatar 360 Nicolet FT-IR spectrophotometer in the range of 4000–500 cm⁻¹ by preparation of KBr discs. Sample analysis was carried out using the E2 OMNIC

software. Mass spectra (TOF-ES) were recorded on a Waters 2795 separation module/micromass LCT platform.

The lower critical solution temperatures (LCST) of the copolymer dispersions (3 mg mL⁻¹) in PBS (pH 7.4) were obtained using UV/VIS spectrophotometric measurements (Beckman, DU 800 Spectrophotometer). Absorbance of polymer dispersions at 550 nm was monitored as a function of temperature (one heating cycle at rate of 1 °C min⁻¹). The same method was used for co-polymer dispersions in Na₂SO₄ solutions (0.05, 0.1 and 0.2 M) to investigate the effect of varying salt concentration on LCST.

Values of hydrodynamic radius of the nanoparticles were determined via scattered light recorded at 90° angle to incident radiation in Dynamic Light Scattering (DLS)) using a Viscotec Model 802 instrument equipped with an internal laser (825–832 nm) with a maximum radiation power of 60 mW. The samples were diluted with filtered, deionized water and at least five measurements of each sample were taken. The mean and standard deviation were calculated. Data processing was performed with the software program OmniSize2.

Zeta potential (ζ) measurements of the nanoparticles were performed by laser Doppler anemometry using a Malvern Zetasizer 2000 equipped with a 10 mW He-Ne laser operating at a wavelength of 633 nm. Measurements were performed in low ionic strength buffer, 1.0 mM HEPES buffer adjusted to pH 7.4. The mean value and standard deviation for each sample was calculated from at least five measurements.

Morphology of the particles was examined using Transmission Electron Microscope (TEM) (Jeol Jem 1010 electron microscope, Japan). A sample of particle suspension was diluted with a solution of phosphotungstic acid (3% w/v, pH 7.4) and observed under TEM. One drop of sample was placed for 1 minute on a copper grid coated with a formvar carbon film. The excess of sample was wicked away with the aid of filter paper prior to imaging by TEM.

The fluorescent intensities were measured at $\lambda_{ex} = 525$ nm, $\lambda_{em} = 553$ nm, slide width 5.0 (Hitachi F-4500 fluorescence spectrophotometer, Hitachi Scientific Instruments, Finchampstead, UK).

Experimental procedures

O-(2-Bromoisobutyryl)tetraethylene glycol

To a dry 100 ml round-bottom flask equipped with a magnetic stirrer bar, tetraethylene glycol (TEG) (10.0 g, 51.5 mmol) and toluene (50 mL) were added, and the resultant solution was evaporated to dryness *in vacuo*. The residual material was re-dissolved in toluene (60 mL) and evaporated to dryness *in vacuo* to give a dried reagent. The dried TEG was then dissolved in anhydrous THF (20 mL) under argon. Triethylamine (TEA) (1.55 g, 15.3 mmol) was added to the mixture and the resulting reaction mixture was cooled in an ice bath, after which 2-bromoisobutyryl bromide (1.78 g, 7.7 mmol) was added in a dropwise manner. The reaction mixture was then allowed to warm to room temperature and was stirred overnight. The reaction mixture was filtered and the filtrate was concentrated *in vacuo* and then purified by silica gel chromatography using diethyl ether as eluent to afford the title compound as a yellowish liquid (2.20 g, 6.4 mmol, 83% yield). ¹H NMR (400 MHz, CDCl₃) δ 1.95 (s, 6H), 3.48 (m, 2H), 3.60-3.63 (m, 2H), 3.65- 3.71 (m, 8H), 3.76 (dd 2nd order, J = 3.39 and 4.84 Hz, 2H), 4.35 (dd 2nd order, J = 3.38 and 4.83 Hz, 2H); HR-MS (ES⁺) calcd for C₁₂H₂₄BrO₆ (MH⁺) 343.0756 and 345.0736, found 343.0925 and 345.0882.

Scheme S1. Synthesis of O-(2-bromoisobutyryl)tetraethylene glycol.



Figure S1. ¹H NMR (400 MHz) spectrum of *O*-(2-bromoisobutyryl)tetraethylene glycol in CDCl₃.



Figure S2. HR-MS of O-(2-bromoisobutyryl)tetraethylene glycol.



Figure S3. IR spectrum of O-(2-bromoisobutyryl)tetraethylene glycol.

Co-polymerization of O-(2-bromoisobutyryl)tetraethylene glycol, lactide and glycolide using ROP method

To a dry 100 ml round-bottom flask equipped with a magnetic stir bar, *O*-(2bromoisobutyryl)tetraethylene glycol (0.18 g, 0.52 mmol), lactide (3.0 g, 21.0 mmol) and glycolide (2.42 g, 21.0 mmol) were added and purged with argon for 20 min. The mixture was melted by heating up to 140 °C. The melt was degassed and purged with Ar for a further 20 min. This was followed by the addition of tin (II) 2- ethyl hexanoate (42 mg, 0.1 mmol). The reaction was left stirring overnight at 140 °C. The resulting polymer was purified by dialysis using acetone (3 x 500 mL) to afford PLGA-Br co-polymer. $M_n = 12$ KDa, PDI = 1.28 as determined by GPC. Molar composition was calculated by ¹H NMR, which gave lactic acid:glycolic acid in 1:1.



Scheme S2. Co-polymerization using ROP method to yield PLGA-Br.



Figure S4. ¹H NMR spectrum of PLGA-Br co-polymer in CDCl₃.



Figure S5. GPC chromatogram for PLGA-Br co-polymer.



Figure S6. IR spectrum of PLGA-Br co-polymer

Preparation of pPoly(lactide-co-glycolide)*-block-***poly(poly(ethylene glycol methyl ether methacrylate)**-**co-poly(propylene glycol methacrylate)**)

Block co-polymerization of (PEGMEMA₄₇₅-co-PPGMA₃₇₅) with PLGA-Br using AGET/ATRP method

A round bottom flask fitted with a three-way stopcock was charged with PLGA-Br (1.2 g, 0.1 mmol), PMDTA (17.3 mg, 0.1 mmol) and CuBr₂ (22.3 mg, 0.1 mmol). To the mixture, varying amounts of the two co-monomers, PPGMA and PEGMEMA were used as described in Table S1 to give polymers with different properties. All the materials were dissolved in 15 mL of 2-butanone. The mixture was degassed by flushing with Ar and stirred for 1 h. Tin(II) 2-ethyl hexanoate (40.5 mg, 0.1 mmol) was added as the AGET reducing agent under Ar. The flask was transferred to an oil bath at 60 °C. The polymerization was stopped by opening the flask and exposing the catalyst to air. The reaction mixture was triturated three times using an excess of hexane: diethyl ether (1:1) mixture. The precipitate was then dissolved with chloroform and passed through an aluminum oxide column. The resulting co-polymer was obtained by evaporating to dryness *in vacuo*. The resulting co-polymer at 60% conversion to reduce polydispersity as commercially-supplied PPGMA monomer can contain small amounts of poly(propyleneglycol)dimethacrylate which leads to markedly increased polydispersity and cross-linking at higher conversion.

Polymer	Monomer feed				Molar masses and		ses and	[PPGMA]:[PEGMEMA]
	PPGMA		PEGMEMA		(GPC) / kDa			Molar ratios (⁷ H NMR)
	g	mmol	g	mmol	$M_{ m w}$	M _n	$M_{\rm w}/M_{\rm n}$	
P1	2.11	5.63	0.89	1.87	27.2	20.4	1.3	3:1
P2	1.88	5.00	1.19	2.50	25.6	19.8.	1.3	2:1
P3	1.41	3.75	1.78	3.75	22.3	17.2	1.3	1:1
P4			3.56	7.5	36.3	22.3	1.6	0:1

Table S1. Four polymers with the initial feed amounts, molar masses and distributions, and molar ratios of co-monomers as calculated by analysis of ¹H NMR spectra.



Scheme S3. Block co-polymerization of (PEGMEMA₄₇₅-co-PPGMA₃₇₅) with PLGA-Br using AGET/ATRP method to afford PLGA-*b*-(PPGMA-co-PEGMEMA).



Figure S7. ¹H NMR (400 MHz) spectrum of P1 in CDCl₃.



Figure S8. ¹H NMR (400 MHz) spectrum of P2 in CDCl_{3.}



Figure S10. ¹H NMR (400 MHz) spectrum of P4 in CDCl₃.



Figure S11a. GPC chromatogram of the co-polymer product P2.



Figure S11b – Overlay of GPC chromatograms for PLGA-Br and P2



Figure S12. IR spectrum of the co-polymer product P2.

Preparation of PLGA-*b***·(PPGMA-co-PEGMEMA) nanoparticles**

PLGA-*b*-(PPGMA-co-PEGMEMA) co-polymer was used to produce nanoparticles by interfacial deposition (nanoprecipitation) method. Briefly, the co-polymer was dissolved in acetone (5 mL). The solution was then added dropwise to 10 mL de-ionized water whilst mixing with a magnetic stirrer. Stirring was continued at ambient temperature until complete evaporation of the organic solvent had taken place. The resulting nanosphere dispersion was passed through a 1.2 μ m filter. Conventional PLGA nanoparticles were prepared using exactly the same method for comparison purposes.

Rhodamine 6G (50 mg) labelled nanoparticles were prepared using the same method. The mixture was covered with aluminum foil. After filtration, the suspension was passed through PD-10 desalting column with Sephadex G-25 (Amersham Biosciences) to remove unencapsulated fluorescent dye.

Colloidal stability of nanoparticle dispersions

The stability of nanoparticle dispersions was monitored by measuring their turbidity as a function of the electrolyte concentration. Sodium sulfate solution (1.5 ml, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.45, 0.50, 0.55, 0.60 and 0.80 mol L^{-1}) was added to the nanoparticle dispersions (0.5 mL, 0.2% w/v) and the turbidity of the dispersions was measured after 10 min (UV/VIS spectrophotometer - Beckman, DU 800). The critical coagulation concentration (CCC) was determined from a plot of turbidity versus salt concentration.



Figure S13. Determination of the critical coagulation concentration (CCC) of PLGA and PLGA-*b*-(PPGMA-co-PEGMEMA) NPs, assessed by the turbidity method.

As shown in Fig. S13, the addition of very small concentration of sodium sulfate led to an increase in turbidity of PLGA nanoparticles (i.e. $CCC \le 0.05 \text{ mol } L^{-1}$) indicating the formation of aggregates. On the other hand, PLGA-*b*-(PPGMA-co-PEGMEMA) nanoparticles showed higher stability with increasing salt concentration, (i.e. $CCC = 0.45 \text{ mol } L^{-1}$). This indicated the effective steric hindrance provided by (PPGMA-co-PEGMEMA) chains.

As expected the PLGA nanoparticles were stabilized by electrostatic repulsion forces induced by the presence of free terminal carboxylic acid groups, rendering sensitivity to electrolyte concentration or change in pH.⁽¹⁾ In contrast, PLGA-*b*-(PPGMA-co-PEGMEMA) nanoparticles were sterically stabilized by the hydrophilic corona.

	Zeta Potential± S.D (mV)				
Temperature (°C)	PLGA NPs	PLGA- <i>b</i> -(PPGMA-co- PEGMEMA) NPs			
25	$-48.2 \pm 2.1 \text{ (exp76)}$	-1.6 ± 1.9			
42	-54.9±16.0	-1.5 ± 3.6			

Effect of temperature on Zeta potential

Effect of temperature on particle size

Effect of temperature on particle size was determined by DLS. Samples were prepared using the nanoprecipitation method, filtered through a 1.2 μ m filter, and resuspended in PBS (pH 7.4). Heating/cooling cycles were carried out and measurements were performed at different temperatures (37 and 41 °C) in PBS (pH 7.4).

Determination of thermal transition temperature for nanoparticles via turbidity measurements

The change of turbidity of P2 nanoparticles suspensions (in PBS, pH 7.4) in response to change of temperature was investigated using UV/VIS spectrophotometer (Beckman, DU 800). Absorbance measurements were taken at 550 nm. Several heating/cooling cycles were performed to check the reversible thermal transition for nanoparticles. Cycles were programmed to run from either 20–60 $^{\circ}$ C (ramp rate of 1 $^{\circ}$ C min⁻¹) or 37–42 $^{\circ}$ C (ramp rate of 5 $^{\circ}$ C min⁻¹).



Figure S14. Reversible change of P2 suspension turbidity in response to change of temperature (seven heating/cooling cycles T = 37-42 °C, ramp rate = 5 °C min⁻¹), assessed by UV-VIS at 550 nm.

Time course experiments were conducted to investigate the change of turbidity of nanoparticles with time. Absorbance measurements of nanoparticles suspension in PBS (pH 7.4) at 550 nm were

taken for a period of three minutes at specific temperatures (i.e. 37, 38, 39, 40, 41 and 42 °C) using UV/VIS spectrophotometer (Beckman, DU 800).

To investigate the tunable transition of temperature for these systems, **P1**, **P2**, **P3** and **P4** copolymers with LCSTs of 30, 39, 53 and 82 °C respectively were prepared. Each of these polymers was used to prepare nanoparticles. LCST of each polymer was monitored along with the thermal transition temperature (TTT) for nanoparticles turbidity using the same procedure mentioned above. These measurements were taken in PBS (pH 7.4) and sodium sulfate solutions (0.05, 0.10 and 0.20 M).



Figure S15. LCSTs of four PLGA-*b*-(PPGMA-co-PEGMEMA) free copolymers and the corresponding TTTs for NPs in PBS (pH 7.4), assessed by UV-VIS at 550 nm.



Figure S16. Effect of varying salt concentration on LCSTs (P1) and TTT (NPs1).



Figure S17. Effect of varying salt concentration on LCSTs (P2) and TTT (NPs2).



Figure S18. Effect of varying salt concentration on LCSTs (P3) and TTT (NPs3).



Figure S19. Effect of varying salt concentration on LCSTs (P4) and TTT (NPs4).

Dip-test assay: probing changes in the surface properties of thermo-responsive NPs

Glass slides were immersed in methanol (15 mL) and silanised by adding (3-chloropropyl) trimethoxysilane (3 mL). The mixture was left for 3 h, transferred to an oven and left at 40 °C overnight. The silanised glass was immersed in Rhodamine 6G labeled nanoparticles suspension at

different temperatures (i.e. 25, 37 and 42 °C) and washed with water. The fluorescent solutions were measured at $\lambda_{ex} = 525$ nm, $\lambda_{em} = 553$ nm (Hitachi F-4500 fluorescence spectrophotometer).

TEM Analysis



Figure S20 TEM images of (A) PLGA NPs and (B) PLGA-B-(PPGMA-co-PEGMEMA) NPs.

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