Lipidated polymers for the stabilization of nanostructured drug delivery vehicles

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MATERIALS AND METHODS

Materials.

Tris(2-(dimethylamino)ethyl) amine (Me_6TREN) was synthesized according to literature procedures.[1] Dichloromethane (DCM, Sigma Aldrich), dimethyl sulphoxide (DMSO, Merck Millipore), copper (II) bromide ($CuBr_2$, Sigma-Aldrich, 98%), dodecyl 2-bromoisobutyrate (DBiB, Sigma Aldrich), α-bromoisobutyryl bromide (BIBB, Sigma-Aldrich), triethylamine (TEA, Sigma Aldrich), 1,2-dilauroyl-*sn*-glycerol (1,2-DLG, Sapphire Biosciences) were used as received. Methyl acrylate (MA, Sigma Aldrich), and poly(ethylene glycol) methyl ether acrylate (PEGMEA, Sigma Aldrich) were de-inhibited by percolating over a column of basic alumina. Copper wire was activated by washing in sulfuric acid for 10 min, followed by the removal of the acid with water and drying of the copper wire.

Cubosome Materials.

Phytantriol (3,7,11,15-tetramethylhexadecane-1,2,3-triol) was a gift from DSM Nutritional Products (Kaiseraugst, Switzerland). Water used in these studies was obtained from a Millipore Milli-Q purification system (Billerica, USA). Phosphate Buffered Saline (PBS) was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride 0.24 g of potassium dihydrogen phosphate and 1.44 g of disodium hydrogen phosphate in sufficient water to produce 1 L and the pH adjusted to 7.4. The salts used to prepare PBS were purchased from Chemsupply (Port Adelaide, SA, Australia). Pluronic F108 was purchased from Sigma-Aldrich (St Louis, MO).

Synthesis of (R)-3-((2-bromo-2-methylpropanoyl)oxy)propane-1,2-diyl didodecanoate (BMPD).

1,2-Dilauroyl-*sn*-glycerol (1.0 g, 2.19 mmol) and triethylamine (1.526 mL, 10.9 mmol) were dissolved in dichloromethane (20 mL). The solution was cooled to 0 °C in an ice bath, and α-bromoisobutyryl bromide (0.5412 mL, 4.38 mmol) was added dropwise. The reaction mixture was warmed to room temperature and stirred for 24 hours. The products were washed three times with 20 mL of deionized water, and the organic layer was passed through a silica gel column to remove any residual bromine and dried over $MgSO_4$. Solids were filtered out, and the solvent was removed under reduced pressure at 45 °C to leave a yellow liquid. Yield: 0.7343 g (55 %)

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.31 (tt, *J* = 5.9, 4.4 Hz, 1H), 4.28 (dddd, *J* = 30.8, 17.7, 11.9, 5.0 Hz, 4H), 2.31 (td, *J* = 7.7, 1.4 Hz, 4H), 1.91 (s, 6H), 1.60 (dd, *J* = 14.6, 7.5 Hz, 4H), 1.26 (d, *J* = 10.4 Hz, 33H), 0.87 (t, *J* = 6.9 Hz, 6H).
^{13}C NMR ($CDCl_3$, δ/ppm): 173.33, 172.92, 171.31, 68.77, 63.75, 62.03, 55.26, 34.33, 34.18, 32.04, 30.80, 30.78, 29.74, 29.59, 29.46, 29.39, 29.25, 29.23, 25.00, 22.80, 14.22. HRMS(ESI$^+$/Q-TOF): m/z = calc. for $[M+Na]^+$, $(C_{31}H_{57}BrO_6Na)^+$: 627.3231, found 627.3237.

Kinetic study polymerization.

MA (0.5 mL, 5.52 mmol, 50 eq.), DMSO (1.0 mL), BMPD (0.0668g, 0.110 mmol, 1 eq.), Me6TREN (0.0047 mL, 0.0177 mmol, 0.16 eq.), $CuBr_2$ (0.0012 g, 0.00552 mmol, 0.05 eq.), were charged to a polymerization flask with a magnetic stir bar and fitted with a rubber septum and the mixture degassed via nitrogen sparging for 15 min after which pre-activated copper wire was carefully added under a nitrogen blanket. The polymerization flask was then resealed and deoxygenated for a further five minutes. Samples were taken at t = 0, 0.17, 0.33, 0.5, 0.67, 0.87, 1, 1.5, 2, 3, 4, and 5 hours for ^{1}H NMR and gel permeation chromatography (GPC), where t0 was when the copper wire was added to the polymerization. The sample for ^{1}H NMR was diluted with $CDCl_3$, while the sample for GPC was diluted with DMAc and passed over a neutral aluminium oxide column to remove any metal salts.
This procedure was repeated using PEGMEA as the monomer, taking time points for the polymerization at t = 0, 0.25, 0.5, 1, 1.5, 2, 3, 5, and 8 hours.

Chain extension of diglyceride-terminated PMA with MA.
MA (0.5 mL, 5.52 mmol, 51 eq.), DMSO (1.0 mL), BMPD (0.0655 g, 0.108 mmol, 1 eq.), Me_6TREN (0.0046 mL, 0.0173 mmol, 0.16 eq.), $CuBr_2$ (0.0012 g, 0.00541 mmol, 0.05 eq.), were charged to a polymerization flask with a magnetic stir bar and fitted with a rubber septum and the mixture degassed via nitrogen sparging for 15 min after which pre-activated copper wire was carefully added under a nitrogen blanket. The polymerization flask was then resealed, deoxygenated for a further five minutes and polymerization was allowed to occur at room temperature for 3 hours.
Samples for NMR and GPC were taken to confirm full conversion. The ^{1}H NMR sample was prepared by dilution with $CDCl_3$, while the sample for GPC was first diluted with DMAc then passed over a neutral aluminium oxide column to remove salts. Once full conversion was confirmed a 1:1 mixture of methyl acrylate (1.67 mL, 18.4 mmol, 170 eq.) and DMSO was deoxygenated and added to the reaction mixture via a deoxygenated syringe. After 24 hours the reaction was stopped and samples taken for ^{1}H NMR and GPC analysis.

Preparation of PPEGMEA diglyceride terminated stabilisers for liquid crystalline drug delivery vehicles.
PEGMEA (0.5 mL, 1.14 mmol, 5 eq.), DMSO (1.0 mL), BMPD (0.1375 g, 0.227 mmol, 1 eq.), Me_6TREN (0.0097 mL, 0.0363 mmol, 0.16 eq.), $CuBr_2$ (0.0025 g, 0.0114 mmol, 0.05 eq.), were charged to a polymerization flask with a magnetic stir bar and fitted with a rubber septum and the mixture degassed via nitrogen sparging for 15 min after which pre-activated copper wire was carefully added under a nitrogen blanket. The polymerization flask was then resealed, deoxygenated for a further five minutes and polymerization was allowed to occur at room temperature for 5 hours.
After 24 hours a sample of the reaction mixture was removed for ^{1}H NMR and GPC analysis. The sample for ^{1}H NMR was diluted with $CDCl_3$, while the sample for GPC was first diluted with DMAc then passed over a neutral aluminium oxide column to remove metal salts. The polymers synthesized were purified by diluting with THF and passing over a neutral aluminium oxide column to remove metal salts and remaining monomer, followed by removal of the solvent by evaporation under a stream of air. The initiator was switched to DBiB under the same conditions.

PEGMEA (0.5 mL, 1.14 mmol, 5 eq.), DMSO (1.0 mL), DBiB (0.0720 mL, 0.227 mmol, 1 eq.), Me_6TREN (0.0097 mL, 0.0363 mmol, 0.16 eq.), $CuBr_2$ (0.0025 g, 0.0114 mmol, 0.05 eq.), were charged to a polymerization flask with a magnetic stir bar and fitted with a rubber septum and the mixture degassed via nitrogen sparging for 15 min after which pre-activated copper wire was carefully added under a nitrogen blanket. The polymerization flask was then resealed, deoxygenated for a further five minutes and polymerization was allowed to occur at room temperature for 5 hours.

Cubosome preparation.

A 1 mL dispersion of cubosomes was prepared by weighing out 10 % (w/v) of phytantriol into a vial. The mixture was then hydrated for 30 minutes in a 1.5% w/v polymer PBS or Pluronic F108 PBS solution before ultra-sonication via a Covaris S220 Focussed-Ultrasonicator (USA) for around 15 minutes with pulsing of 6 seconds on and 2 second off repeatedly for 100 cycles.

Polymer Characterization.

Nuclear Magnetic Resonance (NMR). All NMR spectra were recorded on a Bruker Advance III 400 MHz spectrometer using an external lock and referenced to the residual nondeuterated solvent. Chemical shifts (δ_H) are reported in parts per million (ppm). NMR solvents (CD_3OD and $CDCl_3$) were purchased from Sigma-Aldrich and used as received.

Gel Permeation Chromatography (GPC). GPC analyses of polymer samples were performed using a Shimadzu modular system comprising a DGU-20A3R degasser unit, an SIL-20A HT autosampler, a 10.0 µm bead-size guard column (50 x 7.8 mm) followed by three KF-805L columns (300 x 8 mm, bead size: 10 µm, pore size maximum: 5000 Å), a SPD-20A UV/Vis detector, and an RID-10A differential refractive-index detector. The temperature of columns was maintained at 40 °C using a CTO- 20A oven. The eluent was *N,N*-dimethylacetamide (CHROMASOLV Plus for HPLC) and the flow rate was kept at 1.0 mL min^{-1} using an LC-20AD pump. A molecular weight calibration curve was produced using commercial narrow molecular weight distribution polystyrene standards with molecular weights ranging from 500 to 2 x 10^6 g mol^{-1}. Polymer solutions at approx. 2 mg mL^{-1} were prepared and filtered through 0.45 µm PTFE filters before injection.

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR). ATR-FTIR spectra were recorded from a Shimadzu IR Tracer-100 Fourier Transform Infrared spectrophotometer with an MCT detector by averaging 512 scans at a resolution of 8 cm^{-1} in the MIR region of 4000-400 cm^{-1}.

High resolution mass spectrometry (HRMS). HRMS spectra were recorded on an Agilent 6225 TOF LC/MS Mass Spectrometer coupled to an Agilent 1290 Infinity in ESI mode.

Cubosome Characterization.

Dynamic Light Scattering (DLS). DLS was used to measure the size of the cubosomes and for preliminary stability studies. They were conducted on a Malvern Zetasizer Z. Cubosome dispersions were diluted by a factor of 50,000 into Milli-Q water before measurements were taken at 25 °C.

Small angle X-ray Scattering (SAXS). The internal structure of the cubosomes was assessed using SAXS to characterise the novel surface modified cubosomes and compare them to more traditional cubosomes. All SAXS measurements were performed at the SAXS/WAXS beamline at the Australian Synchrotron. The synchrotron X-ray beam was tuned to a wavelength of 1.127 Å (11.0 keV) at a camera to detector distance of 1034.97 mm which gave the q-range of $0.0176 < q < 1.016$ $Å^{-1}$, where q is the length of the scattering vector defined by $q = (4\pi/\lambda)\sin(\theta/2)$. The q range was calibrated by a silver behenate standard. The 2D SAXS patterns were acquired within 1 s using a Pilatus 1M detector with an active area of 169 x 179 mm^2 and with a pixel size of 172 µm. The cubosome dispersions were transferred to 1.5 mm diameter glass capillaries and loaded into the capillary holder and the SAXS profiles were acquired. The 2D scattering patterns were integrated into the 1D scattering function *I*(q) using the in-house developed software package scatterbrain. Scattering curves are plotted as a function of relative intensity and phase structures are identified by

indexing the Bragg peaks to known relative spacing ratios[2]. The temperature studies utilised a custom capillary holder connected to a water bath to be able to change the sample temperature between measurements.

Cryo-Transmission Electron Microscopy (Cryo-TEM). A laboratory-built humidity-controlled vitrification system was used to prepare the samples for Cryo-TEM. Ambient humidity was approximately 40% for all experiments and ambient temperature was 22 °C. 200-mesh copper grids coated with perforated carbon film (Lacey carbon film: ProSciTech, Qld, Australia) were glow discharged in nitrogen to render them hydrophilic. 4µl aliquots of the sample were pipetted onto each grid prior to plunging. After 30 seconds adsorption time the grid was blotted manually using Whatman 541 filter paper, for approximately 2 seconds. Blotting time was optimised for each sample. The grid was then plunged into liquid ethane cooled by liquid nitrogen. Frozen grids were stored in liquid nitrogen until required. The samples were examined using a Gatan 626 cryoholder (Gatan, Pleasanton, CA, USA) and Tecnai 12 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) at an operating voltage of 120KV. At all times low dose procedures were followed, using an electron dose of 8-10 electrons/Å^2 for all imaging. Images were recorded using a FEI Eagle 4kx4k CCD camera at magnifications ranging from 15 000x to 50 000x. The Fourier transform was generated by the FTT function in the software program ImageJ from the cryo-TEM images.

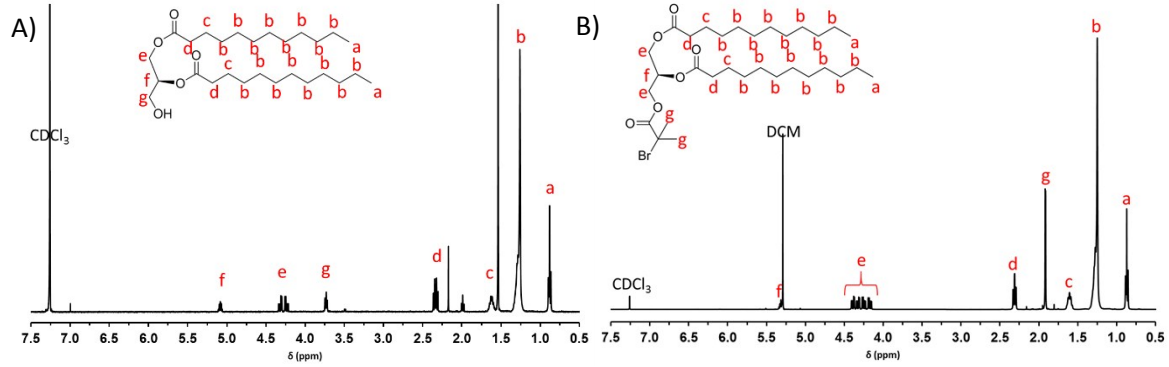

Figure SI 1 *^{1}H NMR spectra of A) 1,2-DLG, the precursor to BMPD; and B) BMPD. ^{1}H NMR was recorded in $CDCl_3$*

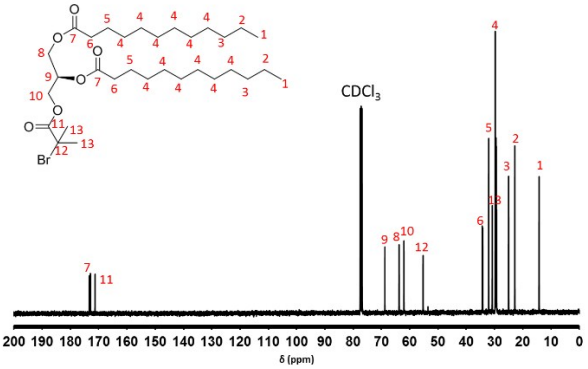

Figure SI 2 *^{13}C NMR spectra of initiator BMPD. ^{13}C NMR was conducted in $CDCl_3$.*

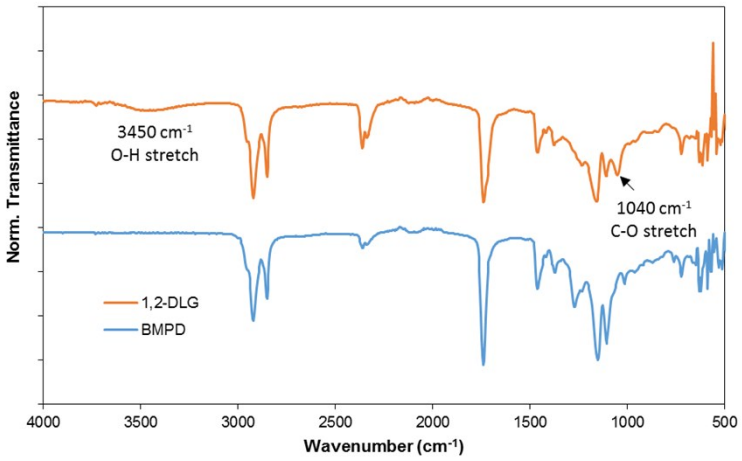

Figure SI 3 *ATR-FTIR spectra of the precursor 1,2-DLG and the diglyceride initiator BMPD*

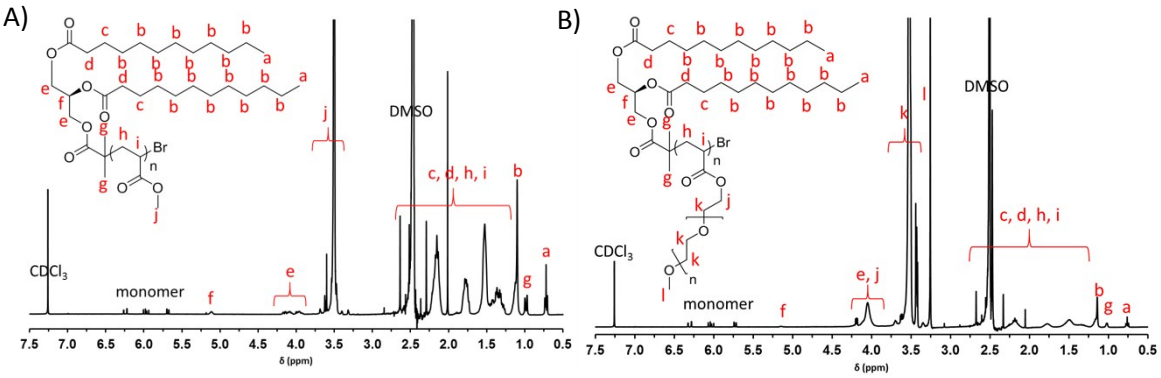

Figure SI 4 *^{1}H NMR spectra of kinetic study of A) PMA at t = 300 min; and B) PPEGMEA at t = 480 min. ^{1}H NMR conducted in $CDCl_3$.*

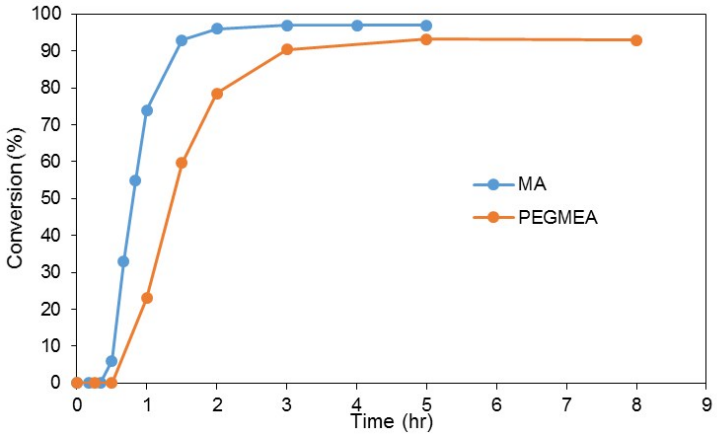

Figure SI 5 *Conversion (%) profile of polymerization of MA ([M] = 3.68 M, [I] = 0.0736 M, [Me6TREN] = 0.0118 M, [CuBr2] = 0.00368 M) and PEGMEA ([M] = 1.14 M, [I] = 0.0227 M, [Me6TREN] = 0.00363 M, [CuBr2] = 0.00114 M) in DMSO at 22 °C. The line is added to guide the reader's eyes.*

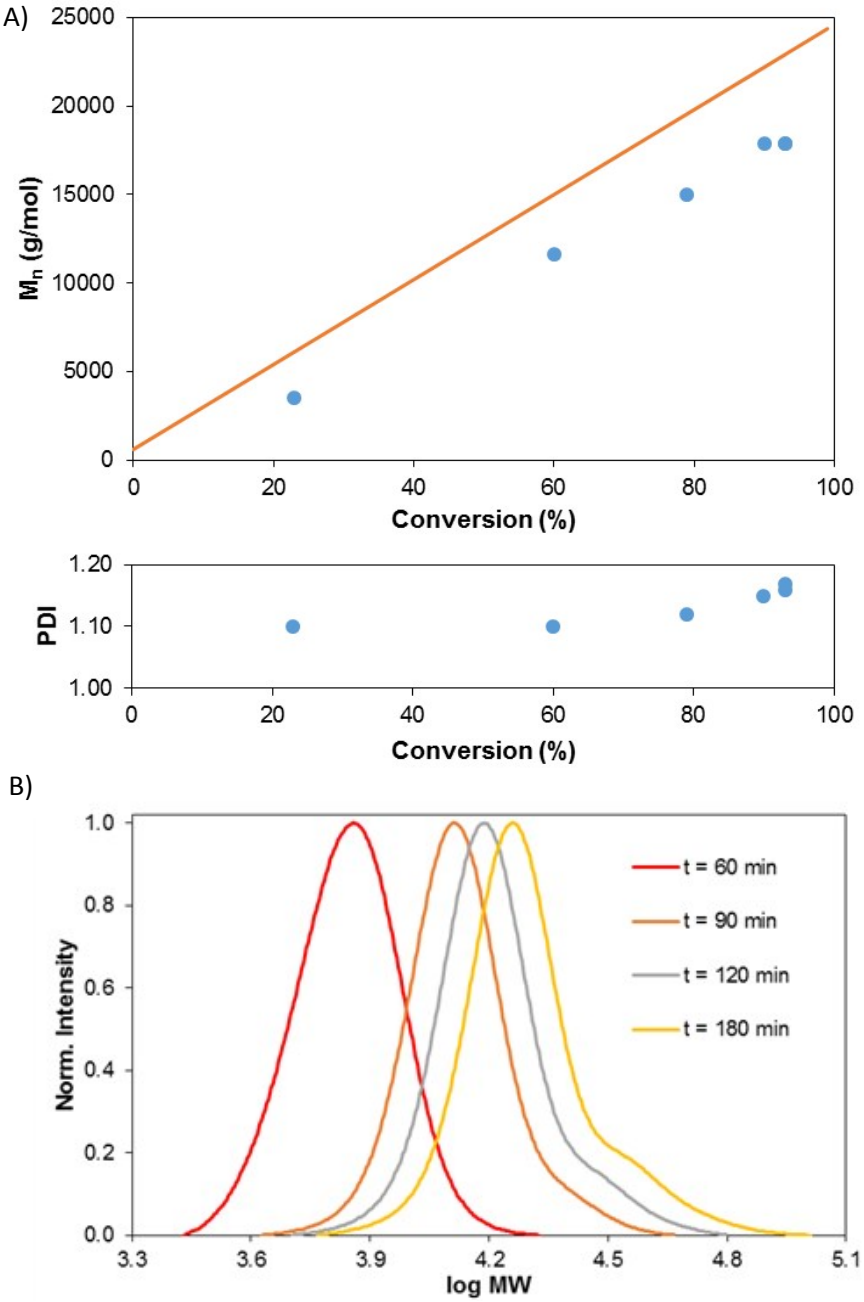

Figure SI 6 *Evolution of molecular weight with conversion for polymerization of PEGMEA ([M] = 1.14 M, [I] = 0.0227 M, [Me_6TREN] = 0.00363 M, [$CuBr_2$] = 0.00114 M) conducted in DMSO at 22 °C. A) M_n against conversion plot and PDI values. The full line represents the theoretically expected molar mass. M_n values were derived from ^{1}H NMR DP calculations by the integration of peak 'a' against peak 'j' from Figure SI 4B. B) GPC spectra of molecular weight distribution over the time of the kinetic study. MWD at time points t = 300 and 480 min were removed due to overlapping distributions.*

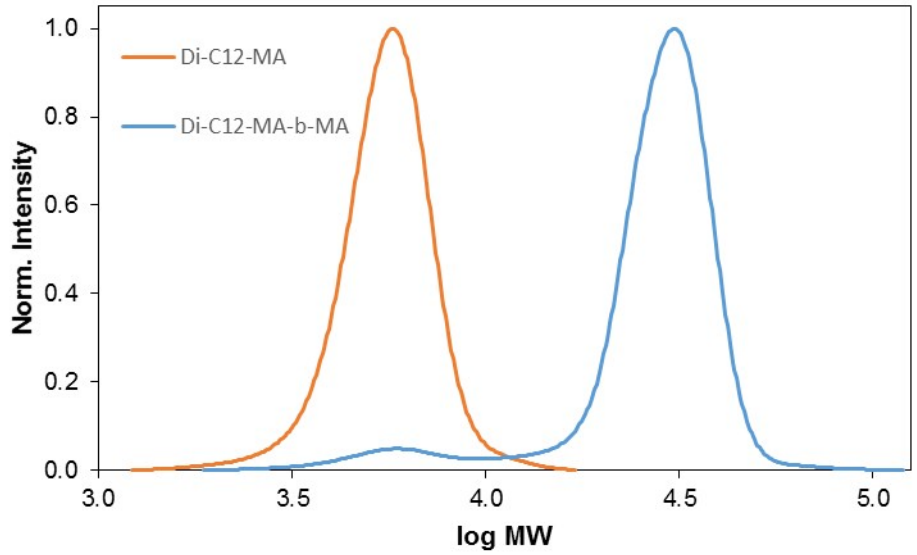

Figure SI 7 *Chain extension of di-C_{12}-terminated methyl acrylate polymer with methyl acrylate*

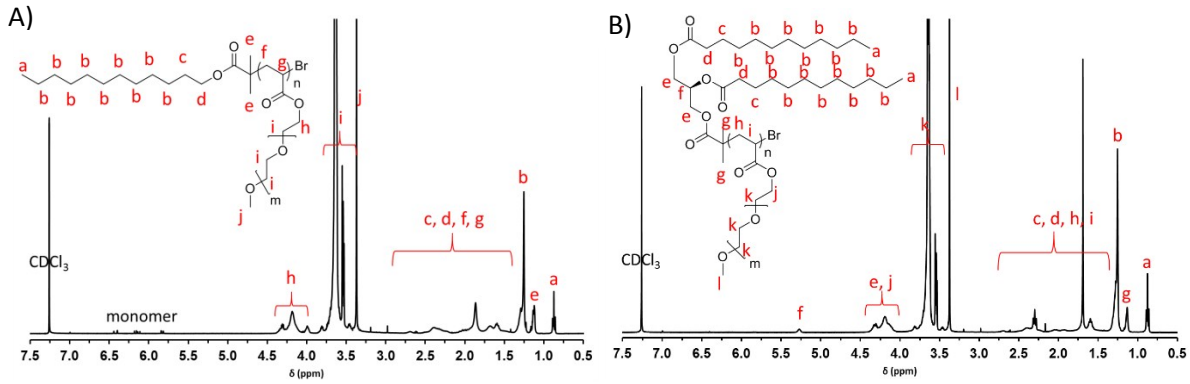

Figure SI 8 *^{1}H NMR spectra of A) C_{12}-terminated PPEGMEA; B) di-C_{12}-terminated PPEGMEA. ^{1}H NMR conducted in $CDCl_3$.*

***Table* SI 1** Polymer Characterization using ^{1}H NMR and GPC

Initiator	Monomer	DP[a]	M_n[a]	M_n[b]	PDI[b]
DBiB	PEGMEA	6	3200	4900	1.10
BMPD	PEGMEA	5	3000	4200	1.11

[a]DP (degree of polymerization) values and M_n were determined by ^{1}H NMR peak integration analysis.
[b]M_n and PDI were determined by GPC analysis in DMAc against polystyrene standards.

Table SI 2 *Table of size measurements of the polymer stabilised cubosomes*

Polymer	Concentration (%w/v)	Average Size (nm)	PDI
C_{12}-terminated PPEGMEA	0.5	247.9	0.281
	1	325.2	0.419
	1.5	632.2	0.434
Di-C_{12}-terminated PPEGMEA	0.5	258.8	0.392
	1	439.2	0.528
	1.5	632	0.454

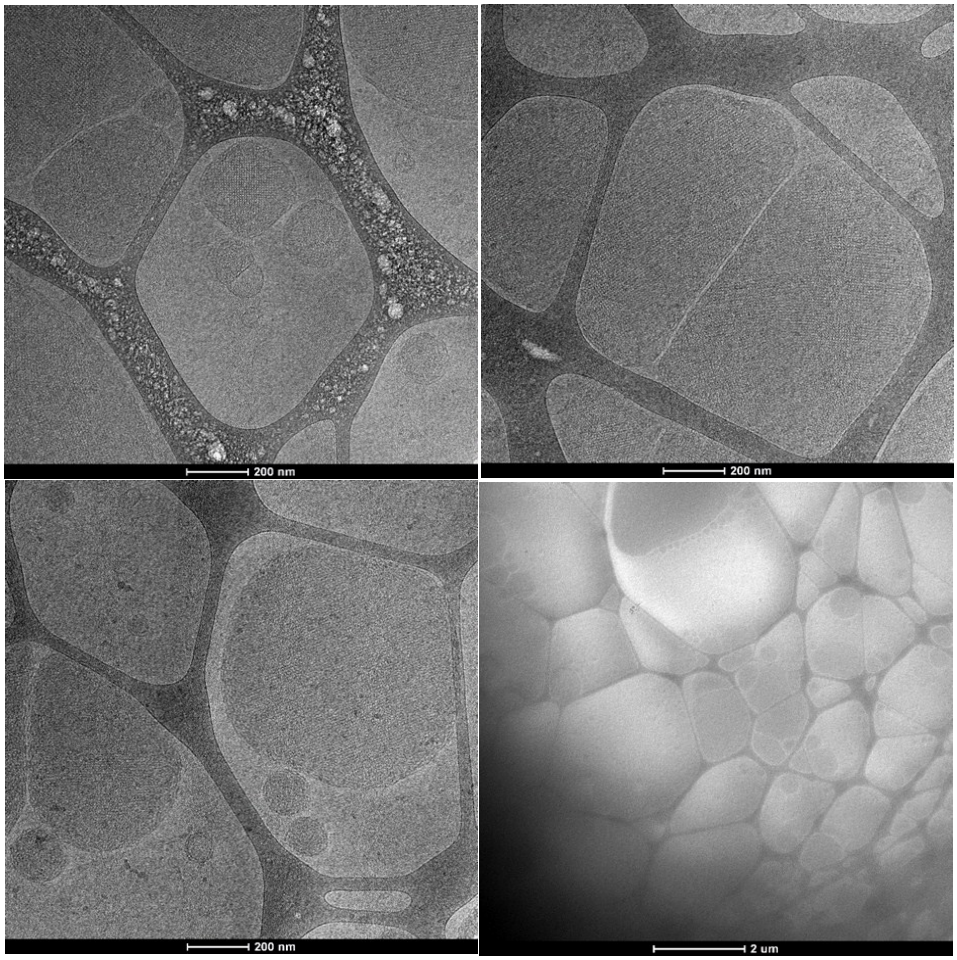

Figure SI 9 *Cryo-TEM images of 1.5% w/v di-C_{12}-terminated PPEGMEA stabilised cubosomes in PBS.*

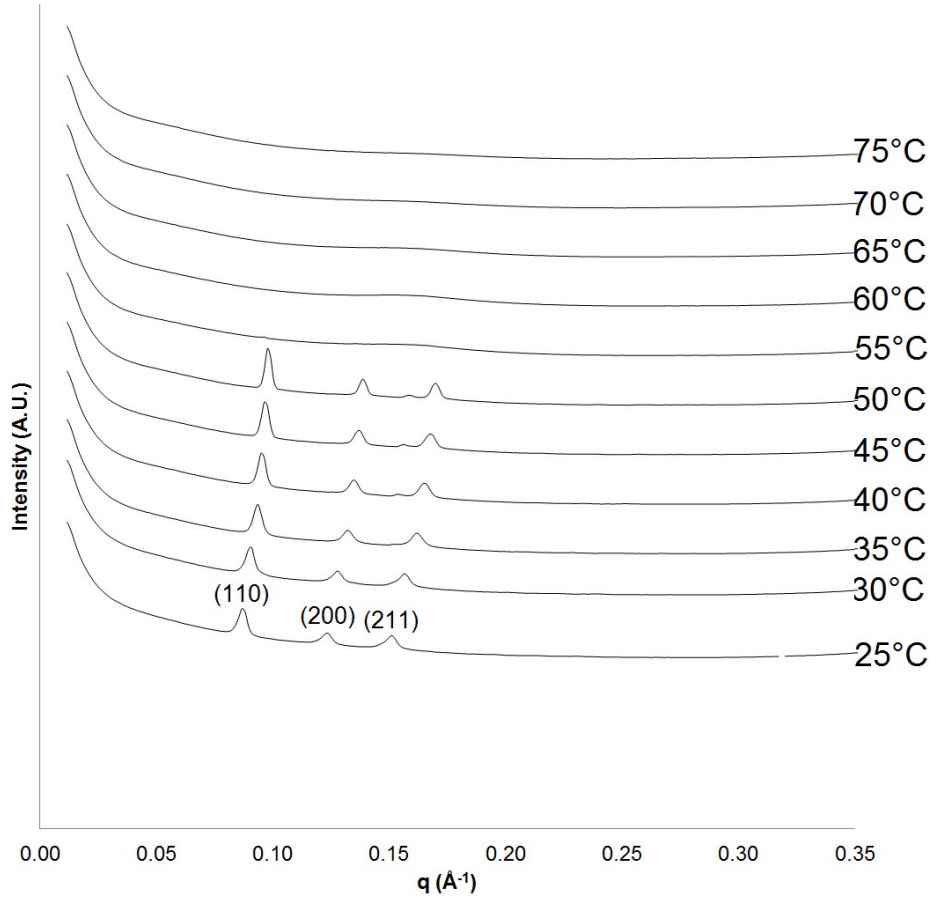

Figure SI 10 *SAXS measurements over a range of temperatures for cubosomes stabilised by 0.5% w/v di-C_{12}-terminated PPEGMEA in PBS.*

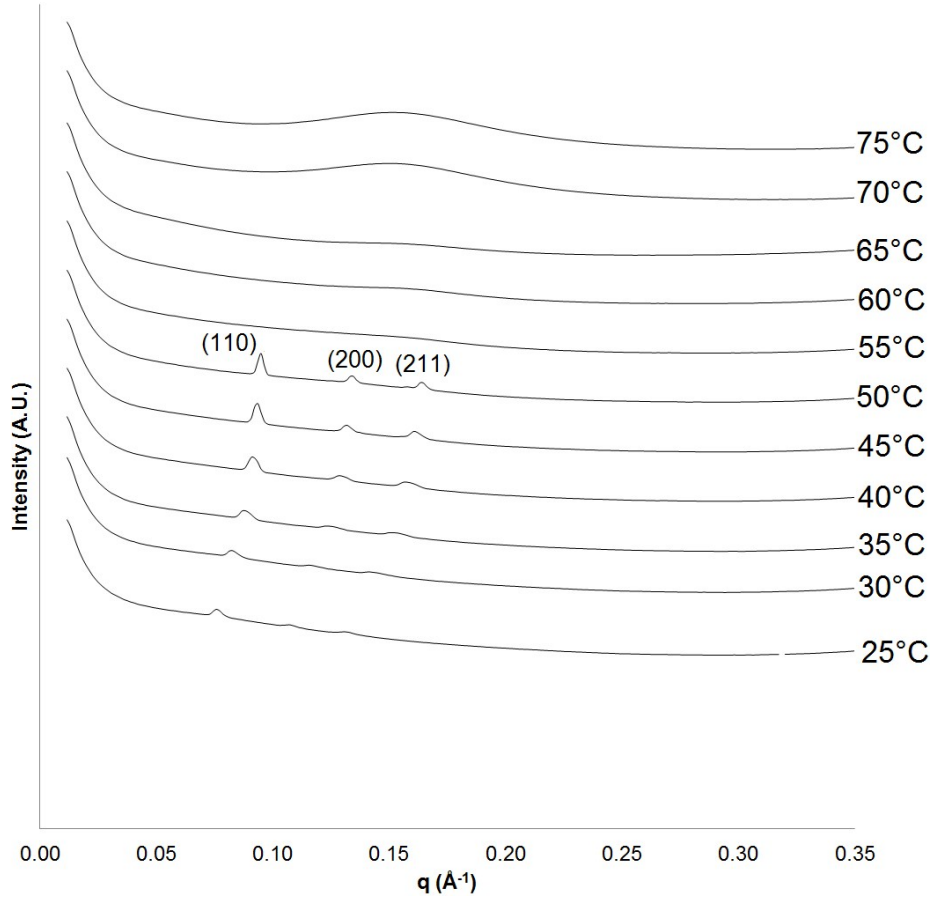

Figure SI 11 *SAXS measurements over a range of temperatures for cubosomes stabilised by 1.0% w/v di-C_{12}-terminated PPEGMEA in PBS.*

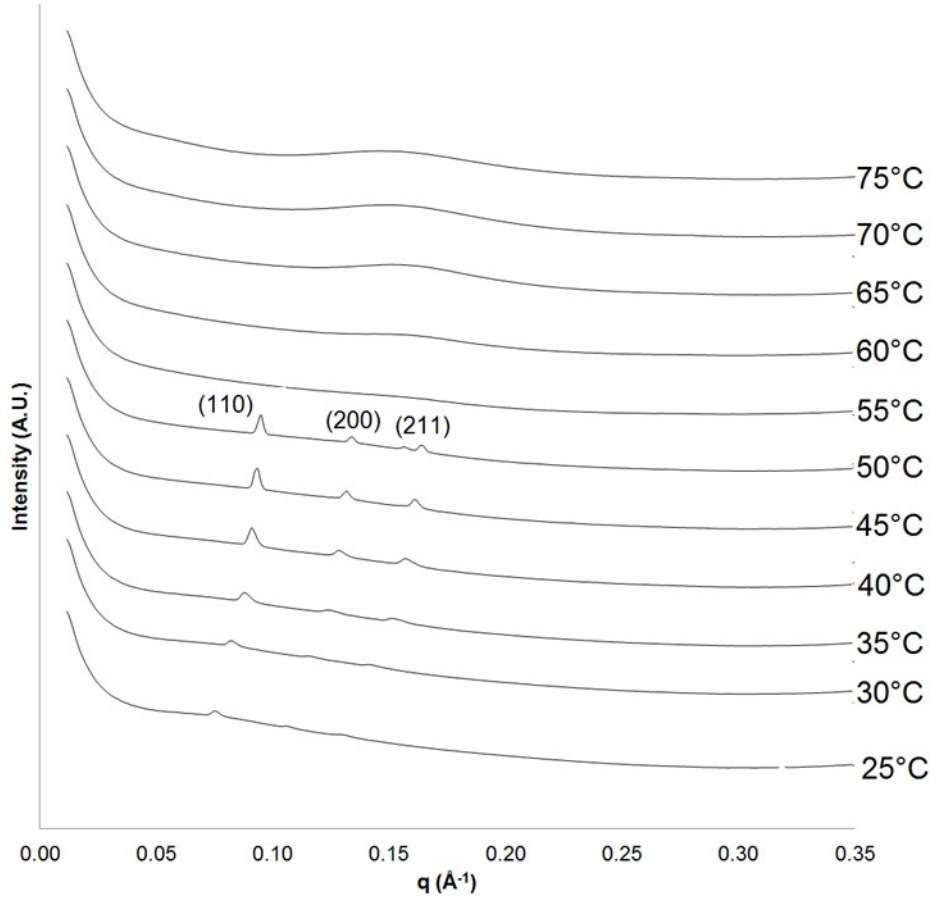

Figure SI 12 *SAXS measurements over a range of temperatures for cubosomes stabilised by 1.5% w/v di-C_{12}-terminated PPEGMEA in PBS.*

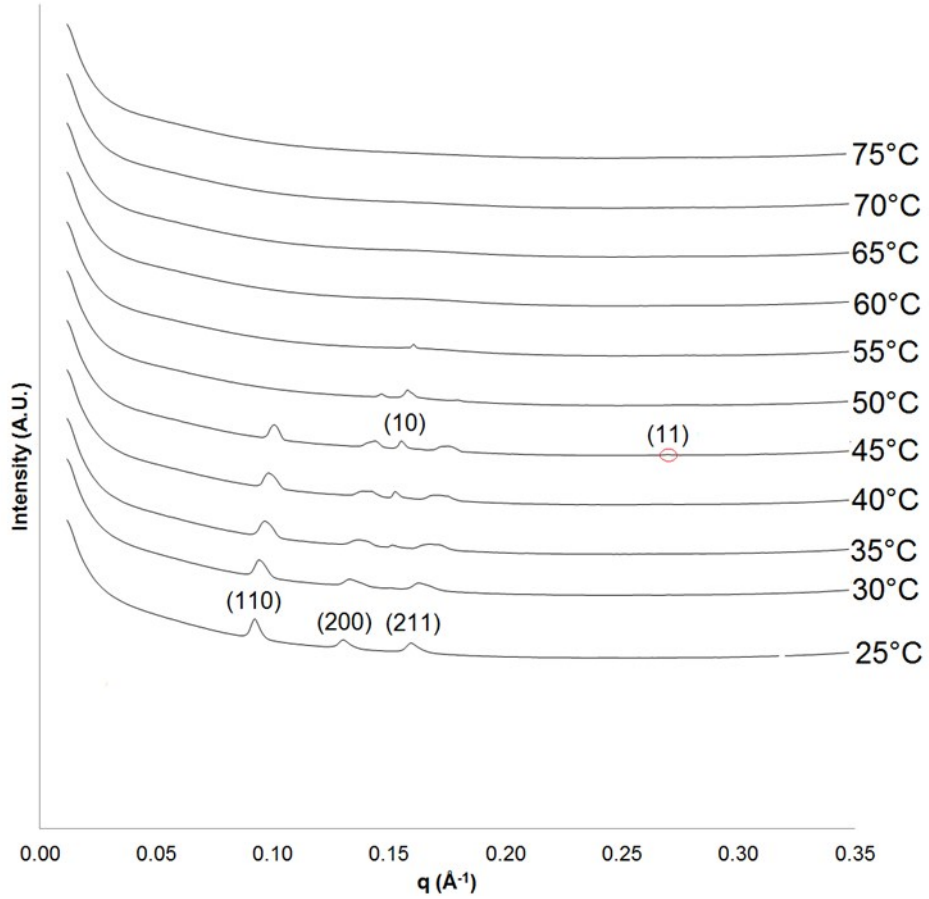

Figure SI 13 *SAXS measurements over a range of temperatures for cubosomes stabilised by 0.5% w/v C_{12}-terminated PPEGMEA in PBS.*

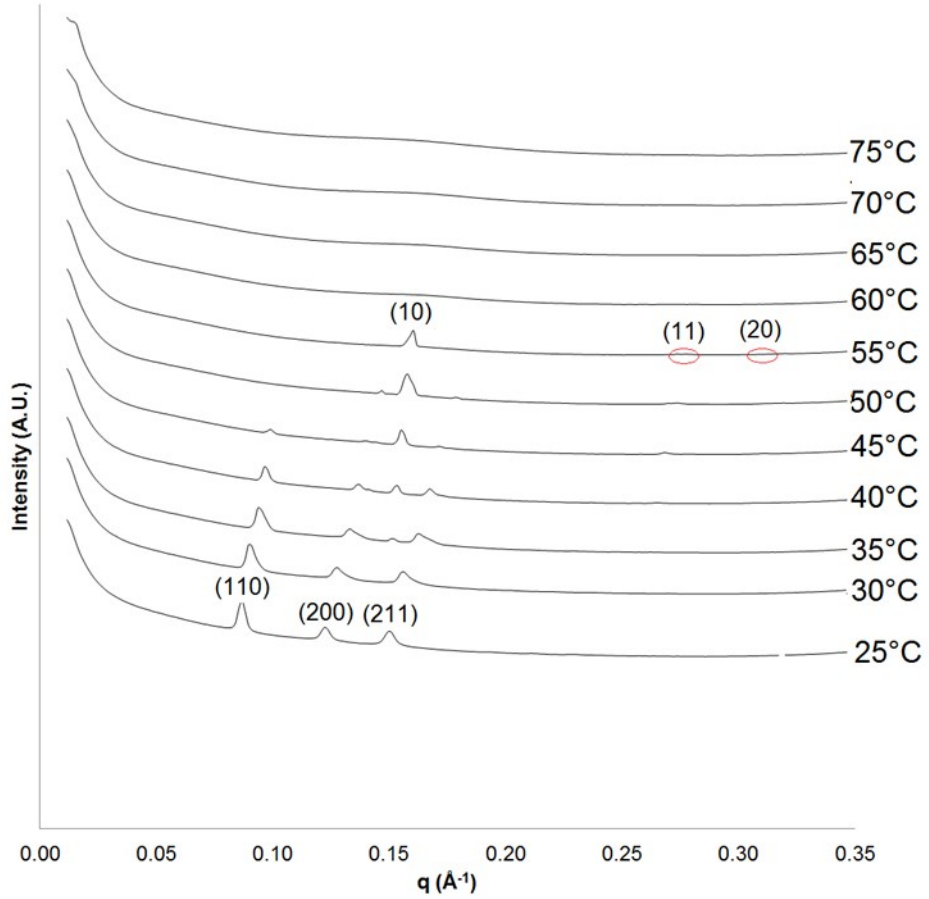

Figure SI 14 *SAXS measurements over a range of temperatures for cubosomes stabilised by 1.0% w/v C_{12}-terminated PPEGMEA in PBS.*

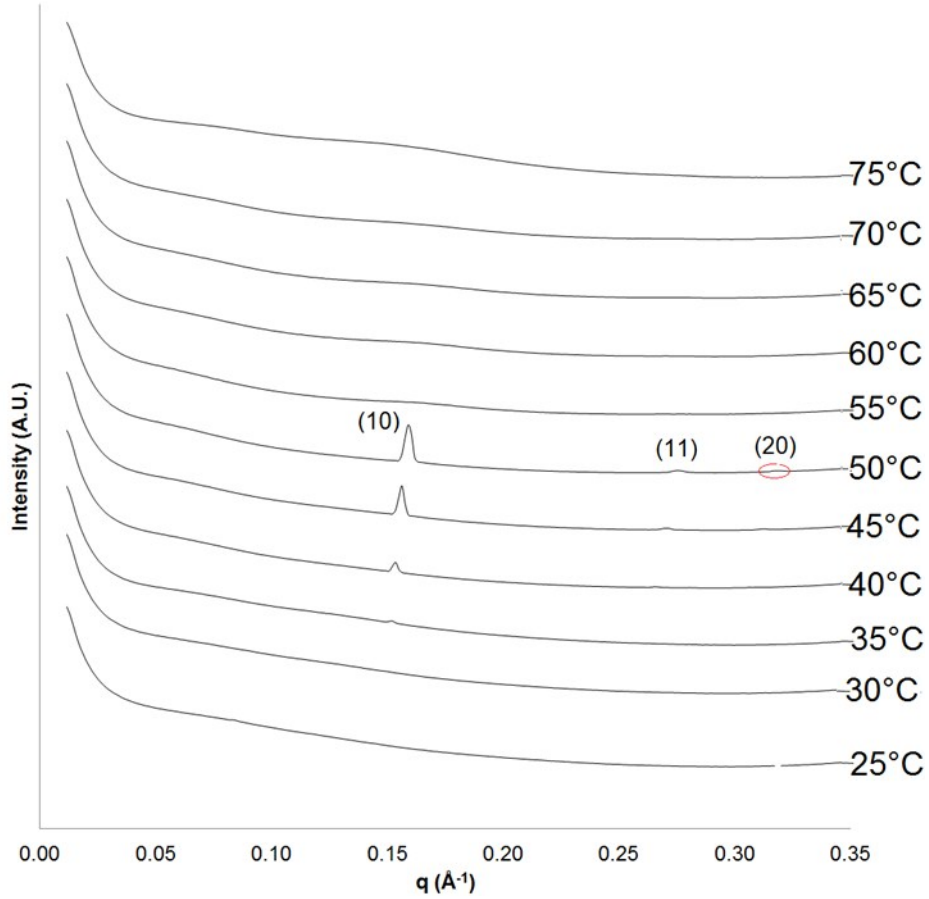

Figure SI 15 *SAXS measurements over a range of temperatures for cubosomes stabilised by 1.5% w/v C_{12}-terminated PPEGMEA in PBS.*

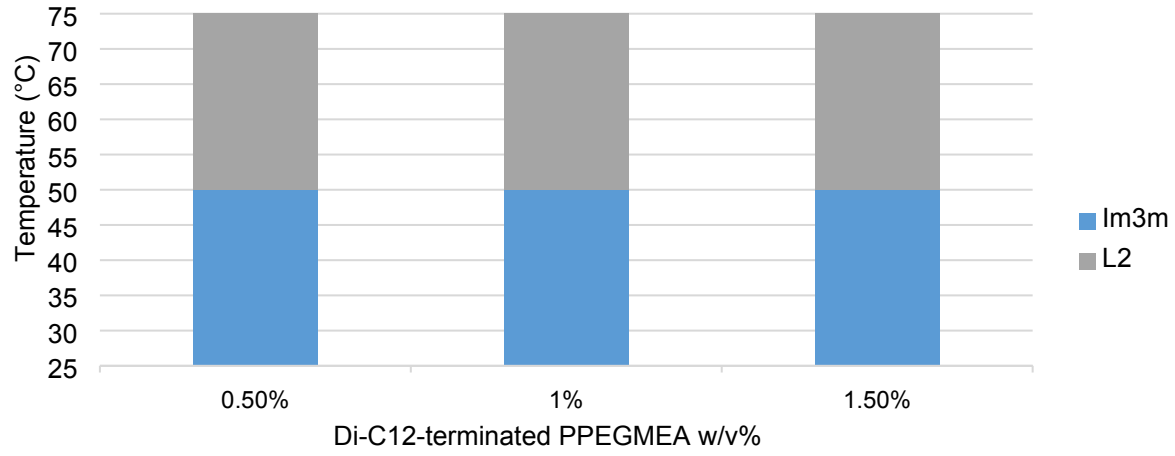

Figure SI 16 *Phase summary of di-C_{12}-terminated PPEGMEA stabilised cubosomes at different w/v% in PBS over a range of temperatures determined from SAXS measurements.*

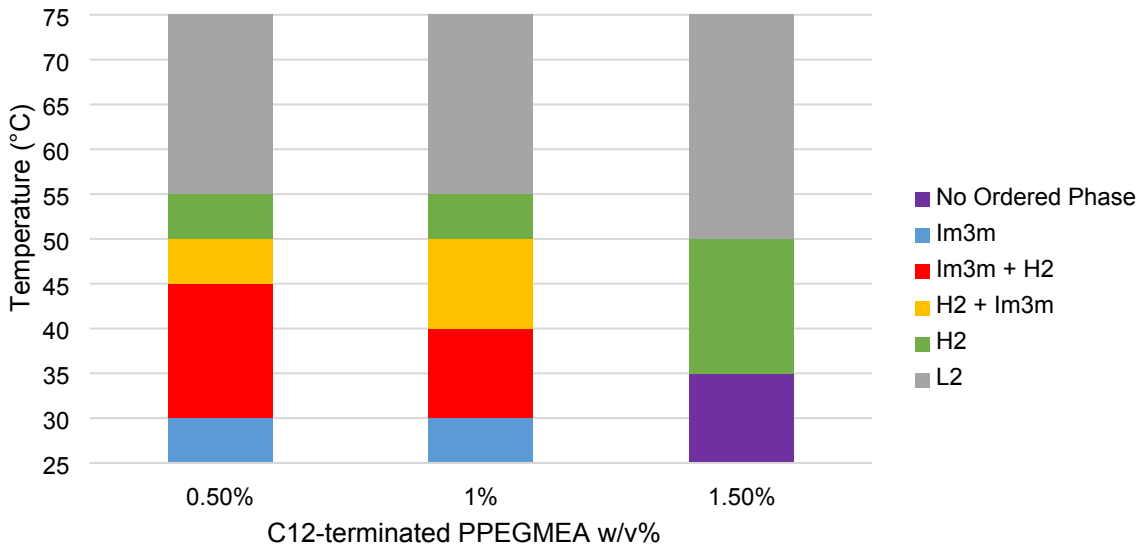

Figure SI 17 *Phase summary of C_{12}-terminated PPEGMEA stabilised cubosomes at different w/v% in PBS over a range of temperatures determined from SAXS measurements. The first phase mentioned in mixed phase systems is the dominant phase*

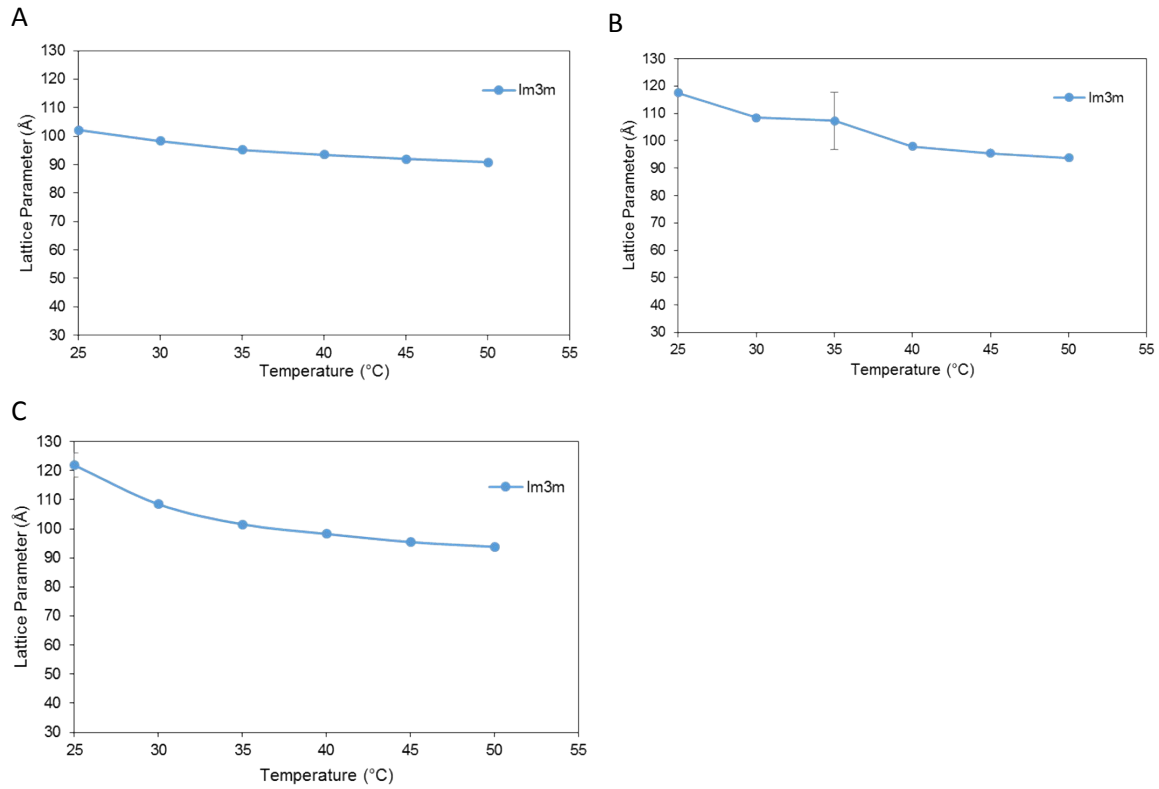

Figure SI 18 *Lattice parameters of di-C_{12}-terminated PPEGMEA stabilised nanostructures at A) 0.5 %w/v; B) 1.0 %w/v; C) 1.5 %w/v in PBS over a range of temperatures*

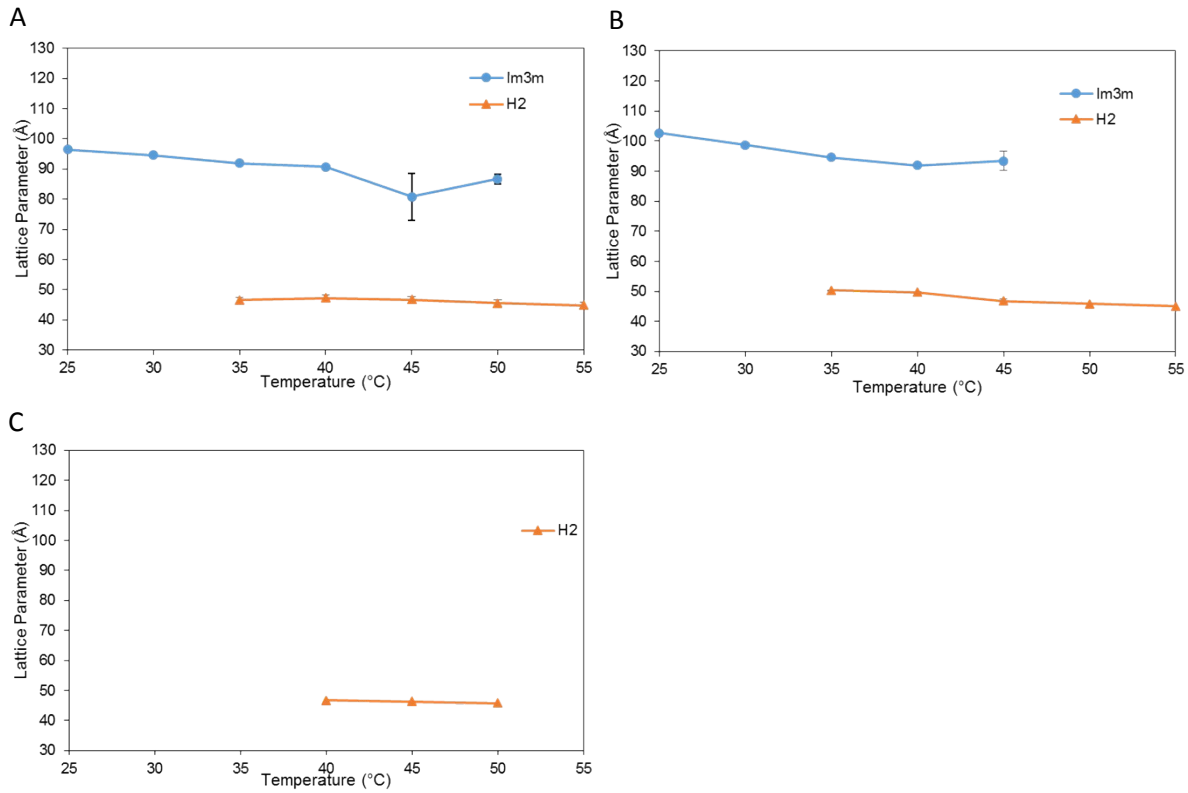

Figure SI 19 *Lattice parameters of C_{12}-terminated PPEGMEA stabilised nanostructures at A) 0.5 %w/v; B) 1.0 %w/v; C) 1.5 %w/v in PBS over a range of temperatures*

References.

1. Ciampolini, M.; Nardi, N., Five-Coordinated High-Spin Complexes of Bivalent Cobalt, Nickel, and Copper with Tris(2-dimethylaminoethyl)amine. *Inorg. Chem.* **1966,** *5* (1), 41-44.

2. Hyde, S.; Ninham, B. W.; Andersson, S.; Larsson, K.; Landh, T.; Blum, Z.; Lidin, S., The Language of Shape. In *The Language of Shape*, Elsevier Science B.V.: Amsterdam, 1997; p iii.