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Novel RAFT amphiphilic brush copolymer steric stabilisers for cubosomes: Poly(octadecyl acrylate)-*block*-poly(polyethylene glycol methyl ether acrylate)

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Copolymers, particularly Pluronic®s, are typically used to sterically stabilise colloidal nanostructured particles composed of lyotropic liquid crystalline bicontinuous cubic phase (cubosomes). There is a need to design and assess new functionalisable stabilisers for these colloidal drug delivery systems. Six amphiphilic brush copolymers, poly(octadecyl acrylate)-*block*-poly(polyethylene glycol methyl ether acrylate) (P(ODA)-*b*-P(PEGA-OMe)), synthesised by reversible addition-fragmentation chain transfer (RAFT), were assessed as novel steric stabilisers for cubosomes. It was found that increasing the density of PEG on the nanostructured particle surface by incorporating a PEG brush design (i.e., brush copolymer), provided comparable and/or increased stabilisation effectiveness compared to a linear PEG structure, Pluronic® F127, which is extensively used for steric stabilisation of cubosomes. Assessment was conducted both prior to and following the removal of the dodecyltrithiocarbonate end-group, by free radical-induced reduction. The reduced (P(ODA)-*b*-P(PEGA-OMe) copolymers were more effective steric stabilisers for phytantriol and monoolein colloidal particle dispersions than their non-reduced analogues. High throughput characterisation methodologies, including an accelerated stability assay (ASA) and synchrotron small angle X-ray scattering (SAXS), were implemented in this study for the rapid assessment of steric stabiliser effectiveness and lyotropic liquid crystalline phase identification. Phytantriol cubosomes stabilised with P(ODA)-*b*-P(PEGA-OMe) copolymers exhibited a double diamond cubic phase (Q_2^D), whilst monoolein cubosomes exhibited a primitive cubic phase (Q_2^P), analogous to those formed using Pluronic® F127.

Introduction

Lyotropic liquid crystalline nanostructured particles, such as cubosomes, are of significant interest due to their well-defined, ordered internal structure. These self-assembled structures possess a high volume fraction of lipid and large internal surface area for loading with both hydrophilic and lipophilic therapeutics and biomedical imaging agents.[1-7] Lipids, such as phytantriol and monoolein (GMO) (structures in Figure 1) are common examples of building blocks for these lyotropic liquid crystalline phase systems.[8] As amphiphiles with both lipophilic and hydrophilic domains, these lipids readily self-assemble in aqueous environments.[9-11] However, lyotropic liquid crystalline nanostructured particles in aqueous solutions can only be colloidally stable for extended periods in the presence of a stabiliser. Currently the range of steric stabilisers for lyotropic liquid crystalline nanostructured particles remains limited, with the most commonly employed stabiliser being Pluronic® F127.[12-17]

Pluronic® F127 ("F127") is an amphiphilic triblock copolymer, with a number average molar mass of 12 600, consisting of 100 units on average of polyethylene glycol (PEG) on both sides of a 65-unit long polypropylene oxide (PPO) block (Figure 1). F127 has been extensively used for sterically stabilising lyotropic liquid crystalline bicontinuous cubic nanostructured particles. Although F127 is an effective steric stabiliser for cubosomes, it may not always be the most effective steric stabiliser available for different lipids.[17, 18] Alternative steric stabilisers known for stabilising lyotropic liquid crystalline colloidal particles include β-casein[19], silica particles[20], Laponite[21], modified cellulose[22], ethoxylated phytosterol[23], Polysorbate 80[24], Pluronic® F108[17] and Myrj® 59[25]. Discovery of the alternative steric stabilisers: Pluronic® F108 and Myrj® 59, were enabled by the development of high-throughput methodologies, which has facilitated implementation of high-throughput preparation and screening protocols.[17, 25-27]

Previous studies on steric stabilisers possessing different architectures have shown varying degrees of effectiveness of steric stabilisation.[17, 18, 25, 28] In previous work, an amphiphilic brush copolymer,

poly(octadecyl acrylate)-*block*-poly(polyethylene glycol methyl ether acrylate) (P(ODA)-*b*-P(PEGA-OMe)) was synthesised, using reversible addition-fragmentation chain transfer (RAFT) polymerisation (Figure 1 and Table 1).[29, 30] These P(ODA)-b-P(PEGA-OMe) copolymers showed self-assembly behaviour in excess water and were noted to be potential candidates for self-assembled drug delivery systems, such as micelles.[30] Although the amphiphilic 'brush' copolymer structure has to our knowledge never been reported for use in the steric stabilisation of lyotropic liquid crystalline nanostructured particles, a polymer brush structure with branching arms of PEG was thought to be an effective design for steric stabilisers because of reports that non-linear structures (i.e., hyperbranched polyglycerols) were advantageous over linear structures. Some of these advantages include antifouling[31, 32], protein resistance[33, 34], less susceptibility to oxidation or thermal stresses than PEG[34], longer plasma half-lives indicating stealth[35-39] properties and prolonging particle circulation[40, 41].

In this study, custom synthesised amphiphilic brush copolymers (P(ODA)-*b*-P(PEGA-OMe)) are assessed for their effectiveness at sterically stabilising cubosomes. The significance of this study is to establish new stabiliser designs/structures that are not commercially available to improve effectiveness of steric stabilisation, whilst validating the use of controlled RAFT synthesised materials for colloidal systems. Synthesising custom steric stabilisers is relatively new, as previous studies in this field/area have predominantly investigated the stability of cubosome dispersions using commercially available surfactants/copolymers (e.g. Pluronic®, Tween®, Myrj®).[17, 25] The advantages of customising and synthesising steric stabilisers are twofold. Firstly having the ability to tune stabilisers will allow opportunities for optimising stabilisation for different lipids. Secondly functionalising stabilisers through the functional groups afforded by the RAFT agent at the end of the hydrophilic domain permits the attachment of targeting moieties (e.g. antibodies or antibody fragments). This will allow the development and use of active targeting systems. Active targeting cubosome systems have not been reported and are important in advancing the use of these colloidal systems for drug delivery applications. The P(ODA)-*b*-P(PEGA-OMe) copolymers were synthesised with the intent to potentially functionalise the terminal RAFT end-group *via* thiol-conjugation to produce actively targeted drug delivery systems. Even though it is possible to functionalise the end of Pluronic® surfactants[42] these copolymers have a unique design (i.e., brush structure), which may be more effective for the steric stabilisation of cubosomes.

Therefore herein we assess the use of these amphiphilic brush copolymers as novel steric stabilisers for cubosomes and compare their effectiveness as stabilisers to the standard control steric stabiliser, F127 (Figure 1 (iii)). Both reduced (RAFT end-group removed; Figure 1 (v)) and non-reduced P(ODA)-*b*-P(PEGA-OMe (Figure 1 (iv)) were assessed. Colloidal particle dispersions prepared using both phytantriol and monoolein (Figure 1 (i) and (ii)) as the core lipids, stabilised with P(ODA)-*b*-P(PEGA-OMe) copolymers were characterised for their effectiveness at providing steric stabilisation using visual assessment and an accelerated stability assay (ASA)[18]. Particle size was determined using dynamic light scattering (DLS) and characterisation of lyotropic liquid crystal internal structure and particle morphology were determined using synchrotron small-angle X-ray scattering (SAXS) and cryo-transmission electron microscopy (cryo-TEM).

Figure 1. Chemical structures of (i) phytantriol, (ii) monoolein, (iii) F127 (control steric stabiliser), (iv) P(ODA)-*b*-P(PEGA-OMe) (non-reduced) and (v) P(ODA)-*b*-P(PEGA-OMe) (reduced)

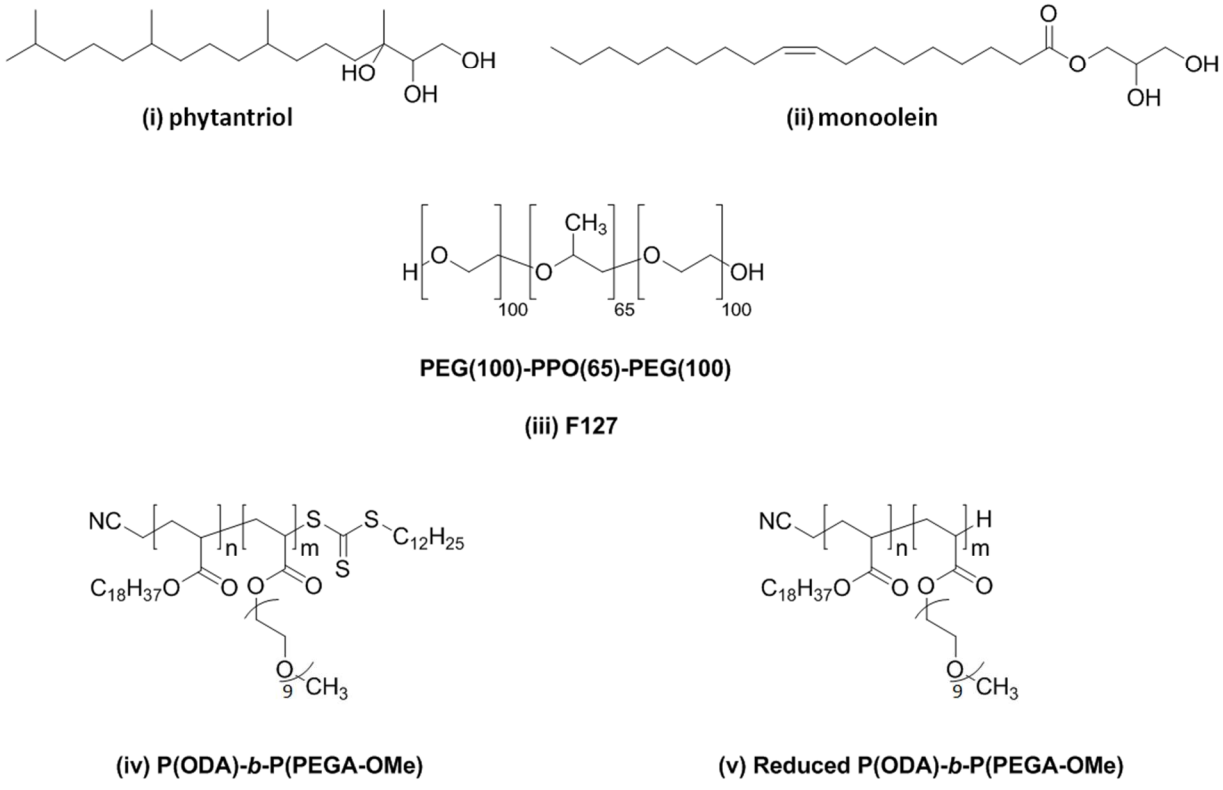

Table 1. Properties of amphiphilic copolymers P(ODA)-*b*-P(PEGA-OMe): number average molar mass (M_n), dispersity (M_w/M_n), [a] Number-average molar mass (M_n) determined by ^{1}H NMR [b] Number-average molar mass (M_n) and dispersity (M_w/M_n) determined by chloroform gel permeation chromatography, with the molar mass in polystyrene equivalents. Reproduced from Ref [30]

Polymer P(ODA)-*b*-P(PEGA-OMe)	M_n Theory	M_n[a] NMR	M_n[b] GPC	M_w/M_n[b] GPC
$P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{27}$	14500	15200	15400	1.33
$P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{35}$	19400	19000	17500	1.33
$P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{39}$	20900	20700	17900	1.32
$P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{23}$	17500	14600	14100	1.46
$P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{31}$	22100	18200	15700	1.42
$P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$	23700	19600	16700	1.41

Materials and methods

Materials

Phytantriol (3,7,11,15-tetramethylhexadecane-1,2,3-triol) was obtained from DSM Nutritional Products, NSW. Monoolein (1-oleoyl-*rac*-glycerol ≥99%), Pluronic® F127, fluorescein sodium salt and 0.01 M phosphate buffered saline (PBS) solution (pH 7.4) were purchased from Sigma-Aldrich, NSW. P(ODA)-*b*-P(PEGA-OMe) amphiphilic brush copolymers, in their non-reduced and reduced states, were synthesised by RAFT polymerisation.[30]

Methods

Preparation of Lyotropic Liquid Crystalline Bicontinuous Cubic Nanostructured Colloidal Particles

All dispersions contained lipid (phytantriol/monoolein) at 50 mg lipid per 500 μL of aqueous phase. Control lyotropic liquid crystal dispersions were formed using F127 (control steric stabiliser) at 0.3, 0.5, 0.7, 1, 1.2, 1.5 and 2 wt. % of the total sample mass, in 0.01 M PBS buffer solution. The buffer solution was replaced with Milli Q water for cryo-TEM samples. Lyotropic liquid crystal dispersions stabilised with both reduced and non-reduced P(ODA)-*b*-P(PEGA-OMe) copolymers, were made using 0.1, 0.5, 0.7 and 1 wt. % stabiliser concentrations for initial visual assessment and SAXS analysis. For the accelerated stability assay, the molar concentrations of copolymer equivalent to F127 at 0.7, 1, 1.2, 1.5 and 2 wt. % were calculated for reduced P(ODA)-*b*-P(PEGA-OMe) copolymers and used to prepare phyantriol and monoolein dispersions. These dispersions were also analysed using SAXS.

Two methods were used to prepare cubosome dispersions in 1.5 mL Eppendorf tubes. The first method required steric stabiliser to be dissolved in water, before being added to the lipid. This method was used for steric stabilisers with high water solubility: Pluronic® F127 and the reduced P(ODA)-*b*-P(PEGA-OMe) copolymers.

The second method was applied to the less water soluble stabilisers, such as the non-reduced P(ODA)-*b*-P(PEGA-OMe) copolymers, which possessed a dodecyl thiocarbonate RAFT end-group. The second method required the steric stabiliser to be dissolved into the lipid, by dissolving both components together with chloroform. In the second methodology, it is important to remove the chloroform before adding the water or PBS buffer solution to the stabiliser/lipid mix, as the presence of chloroform can affect the quality of the dispersion. This was done by placing these samples in a vacuum desiccator over 14 days to remove the chloroform by evaporation. Full removal of chloroform was verified using ^{1}H NMR, using deuterated methanol as the NMR solvent.

The resulting contents of the tube were sonicated using a probe ultra-sonicator (Misonix Ultrasonic Liquid Processor Microtip Probe Sonicator with a 418 Misonix probe (Misonix Inc., NY, USA)). Two different processing sequences were used for the sonication of cubosome dispersions. For the sonication of phytantriol samples, three programs were processed in succession without any delay time: Program 1 settings: 50 Amplitude, 30 s Process time, 3 s Pulse-time On, 2 s Pulse-time Off; Program 2 settings: 45 Amplitude, 1 min Process time, 2 s Pulse-time On, 4 s Pulse-time Off; and Program 3 settings: 40 Amplitude, 1 min Process time, 2 s Pulse-time On, 4 s Pulse-time Off. The sequence resulted in a total sonication time of 2.5 min per sample.

For the sonication of monoolein samples the three programs were: Program 1 settings: 50 Amplitude, 1 min Process time, 1 s Pulse-time On, 10 s Pulse-time Off; Program 2 settings: 45 Amplitude, 1 min Process time, 1 s Pulse-time On, 10s Pulse-time Off; and Program 3 settings: 40 Amplitude, 1 min Process time, 1 s Pulse-time On, 10 s Pulse-time Off. The sequence resulted in a total sonication time of 3 min per sample. The sample temperature during sonication was measured using a temperature probe and monitored to prevent overheating of samples. The sample sonication temperature was observed to be consistent between 65 °C to 70 °C, during pulse sonication of samples.

Characterisation of the effectiveness of the copolymer stabilisers

The P(ODA)-*b*-P(PEGA-OMe) copolymers were assessed for their ability to perform as steric stabilisers by visual assessment and an accelerated stability assay.[18] The protocol used for ASA was developed to distinguish between fair to excellent stabilisers that passed the initial visual assessment of particle stability. Only dispersions given a visual assessment score of +++ out of +++, which is the typical scoring for milky, aggregate-free dispersions, equivalent in appearance to the dispersions prepared using control stabiliser F127 at 1 wt. % stabiliser concentration, were assessed with ASA.[18]

Briefly, phytantriol dispersions were mixed at equal volumes with dye solution (i.e., 15 μL cubosome sample mixed with 15 μL dye) and pipetted into a 384 black round well Corning® plate. The same was prepared using PBS buffer solution instead of dye solution (3.1×10^{-4} mg/mL) for control samples. Negative control samples consisted of PBS and dye. Each cubosome mixture with dye or PBS buffer was prepared in triplicate. Fluorescence signal intensities were taken pre and post-centrifugation. The centrifugation of plates was performed with a Heraeus Multifuge ×3 Centrifuge (Thermo Scientific, Germany). Fluorescence signal measurements were taken using FlexStation3 Multi-mode Microplate Reader (Molecular Devices Company, CA, USA), and processed on SoftMax Pro software. The plate was initially centrifuged at 645 xg (1800 rpm) for 5 min, measured for fluorescence signal and then re-centrifuged

at 796 xg (2000 rpm) for 5 min. Phytantriol dispersions stabilised with F127 at 0.3, 0.5, 0.7, 1 and 1.2 wt. % were used as a control standard for the ASA.

The intensity of the fluorescence measured in the ASA is proportional to the quantity of particles dispersed in the solution.[18] Using centrifugation to accelerate the occurrence of particle aggregation within a dispersed sample, poorly and well stabilised systems can be distinguished from samples which appear to be well dispersed. Poorly stabilised systems have greater particle aggregation post-centrifugation and therefore greater changes in fluorescence signal intensities pre- and post-centrifugation. In contrast, good steric stabilisers are able to maintain colloidal stability after centrifugation and therefore have fewer changes in fluorescence signal intensities pre- and post-centrifugation. Thus, lesser changes in fluorescence signal intensities pre- and post-centrifugation indicate greater steric stabiliser effectiveness.[18]

Characterisation of the Internal Structure and Morphology of Lyotropic Liquid Crystal Colloidal Particles

Particle size and polydispersity of dispersed samples was determined by dynamic light scattering using a DynaPro plate reader (Wyatt Technology, Santa Barbara, CA). Data statistics shown for particle size and polydispersity from the DLS instrument are averaged from three repeat measurements. Samples which passed the visual assessment for determining 'good' steric stabilisers were then further analysed using small angle X-ray scattering and cryo-TEM imaging. SAXS is required for establishing the internal phase behaviour (i.e., internal long range order of the crystal lattice) of the dispersed samples under various thermal conditions.

SAXS data was collected at the Australian Synchrotron using a beam with wavelength λ = 1.033 Å (12.0 keV) with a typical flux of approximately 10^{13} photons/s.[43] 2D diffraction patterns were recorded on a Decris-Pilatus 1 M detector of 10 modules. The detector was offset to access a greater q range. A silver behenate standard (lamellar repeat distance of 58.38 Å) was used for calibration. The samples were loaded in 96 well plates and positioned in a custom-designed plate holder capable of holding two plates at a time, within a temperature adjustable sample holder chamber, with the temperature controlled to ± 1.0 °C between 20 and 75 °C. Temperature control was via a recirculating water bath (Julabo, Germany). SAXS was performed on dispersions using a temperature range from room temperature (25 °C) with 5 °C increments to 65 °C. The exposure time for each sample was 0.5 s. SAXS data was analysed using an IDL-based AXcess software package.[44]

Particle structure and morphology was further clarified using cryo-TEM imaging. Samples were prepared in a laboratory-built humidity-controlled vitrification system. Humidity was kept close to 80% 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. Preparation of samples involved 4 µL aliquots of the sample pipetted onto each grid prior to plunging. Samples were left for 30 seconds to be adsorbed onto the grid and excess sample was removed via manual blotting of the grid for approximately 2 seconds, using Whatman 541 filter paper. Blotting time was optimised for each sample. The grid was then plunged into liquid ethane cooled by liquid nitrogen and frozen grids were then 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 120 KV. 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 4k × 4k CCD camera at magnifications between 15000x and 42000x.

Results

Reduced copolymers provide improved colloidal stability over non-reduced copolymers

The amphiphilic brush copolymers, P(ODA)-*b*-P(PEGA-OMe), were able to sterically stabilise phytantriol and monoolein dispersions in both PBS buffer solution and water. The reduced P(ODA)-*b*-P(PEGA-OMe) copolymers were superior to the non-reduced analogues in stabilising phytantriol and monoolein dispersions, creating milky white dispersions with no visible aggregates. These polymers were given the highest visual assessment score (i.e., +++), which was comparable to dispersions stabilised by the standard control stabiliser, F127 (Table 2, 3 and Supplementary Material).

Both phytantriol and monoolein dispersions, stabilised with reduced P(ODA)-*b*-P(PEGA-OMe) copolymers, had an average particle size ranging between 180 and 400 nm in diameter. In contrast, non-reduced P(ODA)-*b*-P(PEGA-OMe) copolymers, which possess a dodecyltrithiocarboante RAFT end-group, produced milky white phytantriol dispersions, with visible aggregates present after sonication. These dispersions were found to have an average particle size ranging between 110 and 340 nm. Furthermore, non-reduced P(ODA)-*b*-P(PEGA-OMe) copolymers were unable to form stable monoolein dispersions. The poor stability of dispersions produced using non-reduced P(ODA)-*b*-P(PEGA-OMe) copolymers is due to the presence of the hydrophobic end-group at the hydrophilic moiety of the P(ODA)-*b*-P(PEGA-OMe) copolymer likely facilitating particle bridging and flocculation.

Table 2. Visual assessment of the stability of phytantriol dispersions using 0.1, 0.5, 0.7 and 1 wt. % P(ODA)-*b*-P(PEGA-OMe) copolymers as steric stabiliser. Key: +++ milky sample with no visible aggregates. ++ milky sample with few visible aggregates, + milky/cloudy sample with aggregates, - translucent sample with large aggregates [not progressed through to ASA or SAXS studies]

			Stabiliser Concentration			
Stabiliser	**ODA**	**PEGA**	**0.1 wt.%**	**0.5 wt.%**	**0.7 wt.%**	**1 wt.%**
P(ODA)$_6$-*b*-P(PEGA-OMe)$_{27}$	**5.6**	**27.1**	-	+	++	++
Reduced			+	++	+++	+++
P(ODA)$_6$-*b*-P(PEGA-OMe)$_{35}$	**5.6**	**35.1**	-	+	++	++
Reduced			+	++	+++	+++
P(ODA)$_6$-*b*-P(PEGA-OMe)$_{39}$	**5.6**	**38.7**	+	+	++	++
Reduced			+	++	+++	+++
P(ODA)$_{10}$-*b*-P(PEGA-OMe)$_{23}$	**9.9**	**23.1**	-	++	++	++
Reduced			+	++	+++	+++
P(ODA)$_{10}$-*b*-P(PEGA-OMe)$_{31}$	**9.9**	**30.7**	-	++	++	++
Reduced			+	++	+++	+++
P(ODA)$_{10}$-*b*-P(PEGA-OMe)$_{34}$	**9.9**	**33.5**	+	++	++	++
Reduced			+	++	+++	+++
Control	**PPO**	**PEG**				
F127	**65**	**100**	+	+++	+++	+++

Table 3. Visual assessment of the stability of monoolein dispersions using 0.1, 0.5, 0.7 and 1 wt. % P(ODA)-*b*-P(PEGA-OMe) copolymers as steric stabiliser.

Stabiliser	ODA	PEGA	Stabiliser Concentration 0.1 wt.%	0.5 wt.%	0.7 wt.%	1 wt.%
$P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{27}$	5.6	27.1	-	-	-	-
Reduced			-	-	++	+++
$P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{35}$	5.6	35.1	-	-	-	-
Reduced			-	-	+++	+++
$P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{39}$	5.6	38.7	-	-	-	-
Reduced			-	-	+++	+++
$P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{23}$	9.9	23.1	-	-	-	-
Reduced			-	-	++	+++
$P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{31}$	9.9	30.7	-	-	-	-
Reduced			-	-	+++	+++
$P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$	9.9	33.5	-	-	-	-
Reduced			-	-	+++	+++
Control	**PPO**	**PEG**				
F127	65	100	-	-	++	+++

Poorly stabilised cubosome systems with visible aggregates have also been previously been reported for using a stabiliser with hydrophobic terminal blocks (i.e. PEG150-distearate).[18] Limitations within the dynamic light scattering equipment may lead to large aggregates being undetected and therefore results from DLS should always be accompanied with the visual assessment of the particle dispersions. Consequently, only phytantriol and monoolein dispersions stabilised using the reduced P(ODA)-*b*-P(PEGA-OMe) copolymers were further assessed using the accelerated stability assay.

Reduced amphiphilic brush copolymers provide comparable steric stabiliser effectiveness to F127

It was generally found that stabilisers that had a longer hydrophobic block (i.e., 10 ODA repeat units) provided greater colloidal stability than stabilisers with a shorter hydrophobic block (i.e., 6 ODA repeat units), for both phytantriol and monoolein dispersions (Figure 2 and Supplementary Materials). This may be due to increased stabiliser affinity to the nanostructured particle through increased hydrophobicity, provided by a longer hydrophobic block.
It is also shown that increasing hydrophilicity within both the 6 and 10 ODA unit copolymer series (e.g., $P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{39}$ and $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$ copolymers respectively) also improved the effectiveness of a stabiliser at providing colloidal stability for both phytantriol and monoolein dispersions (Figure 2 and Supplementary Materials). This increased steric stabilizer effectiveness may be due to increased steric hindrance as a result of increasing the number of units of PEG arms in the copolymer brush structure.

The reduced copolymer that provided the most effective colloidal stability for both lipid systems (i.e., phytantriol and monoolein) was found to be $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$, which showed better or comparable steric stabiliser effectiveness to standard control steric stabiliser, F127 (Figure 2 and Supplementary Materials). This is the amphiphilic brush copolymer stabiliser with the longest hydrophobic (i.e., 10 ODA units) and hydrophilic (i.e., 34 PEGA-OMe units) block in the series.

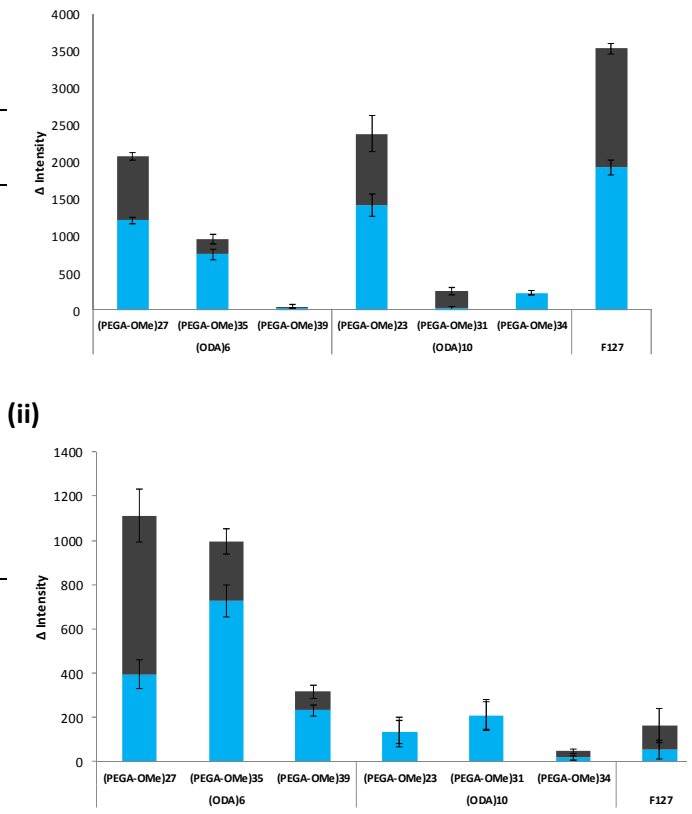

Figure 2. Accelerated stability assay results for (i) phytantriol and (ii) monoolein dispersions stabilised with 1.5 wt. % of reduced: $P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{27}$, $P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{35}$, $P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{39}$, $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{23}$, $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{31}$, $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$ and F127. Samples are initially spun at 645 xg for 5 min and then further spun at 796 xg for 5 min. ASA results after first spin 645 xg are represented by the bottom column (blue color), whilst ASA results after second spin 796 xg are represented by the top column (grey colour) See Supplementary Materials for other ASA results

Reduced copolymers were less disruptive to the lyotropic liquid crystalline bicontinuous cubic phase compared to non-reduced copolymers

Phytantriol dispersions stabilised with 1 wt. % of non-reduced P(ODA)-*b*-P(PEGA-OMe) brush copolymers, presented a mixed Q_2^D cubic and hexagonal (H_2) phase at room temperature (25 °C) (Figure 3 and 4 (i)).

In fact, all phytantriol dispersions, with each of the six non-reduced P(ODA)-*b*-P(PEGA-OMe) brush copolymers at 0.7 and 1 wt. %, possessed the hexagonal (H_2) internal phase at physiological temperature of 37 °C (Figure 4 and Supplementary Materials).

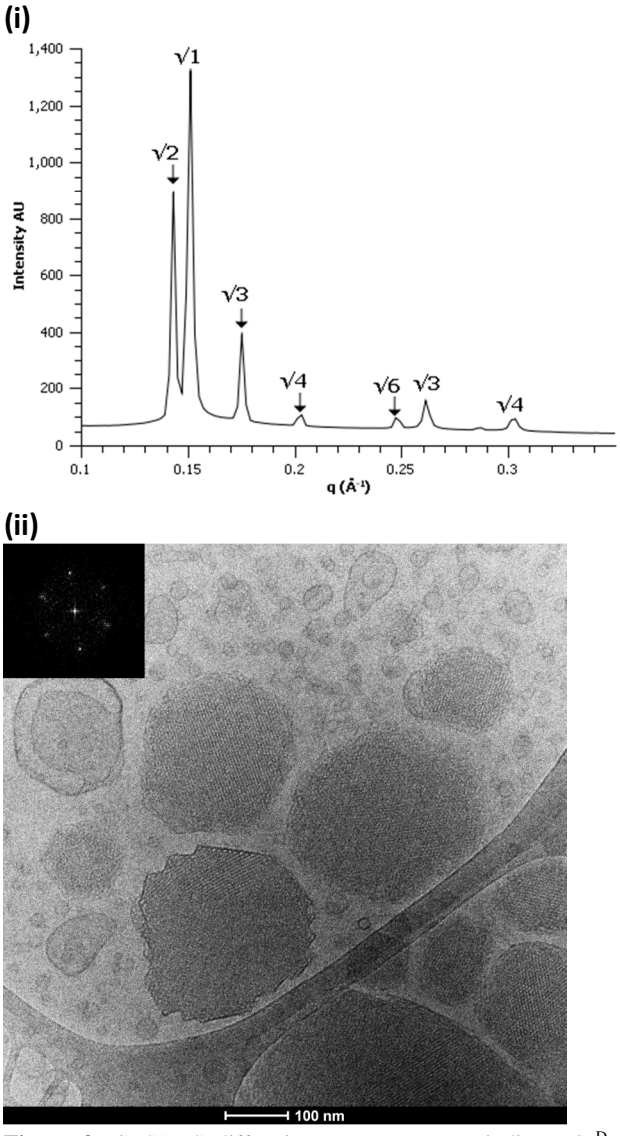

Figure 3. (i) SAXS diffraction pattern (arrows indicate Q_2^D cubic phase and other peaks indicate H_2 hexagonal phase) and (ii) cryo-TEM image observed in the [111] axis plane for phytantriol dispersion stabilised with 1 wt. % non-reduced $P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{39}$ copolymer at 25 °C

In contrast, phytantriol dispersions stabilised with reduced P(ODA)-*b*-P(PEGA-OMe) brush copolymers with various steric stabiliser concentrations (i.e., 0.1, 0.5, 0.7 and 1 wt. % and also with mol equivalent concentrations to F127: 0.7, 1, 1.2, 1.5 and 2 wt. %) were identified to retain the Q_2^D cubic phase at room temperature (25 °C) (Figure 4 and 5). These phytantriol dispersions had a phase transition temperature from Q_2^D to H_2 at 60 °C, identical to that when stabilised using F127 (Figure 4). The Q_2^D phytantriol cubosomes stabilised with 1 wt. % of $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$ had an average lattice parameter of 69.0 Å at 25 °C, which decreased in lattice parameter as the temperature was increased. This result is comparable with literature reports[14, 17, 19, 25] of phytantriol systems stabilised by F127 at the same concentration (e.g., lattice parameter of 68.6 Å at 25 °C)[14].

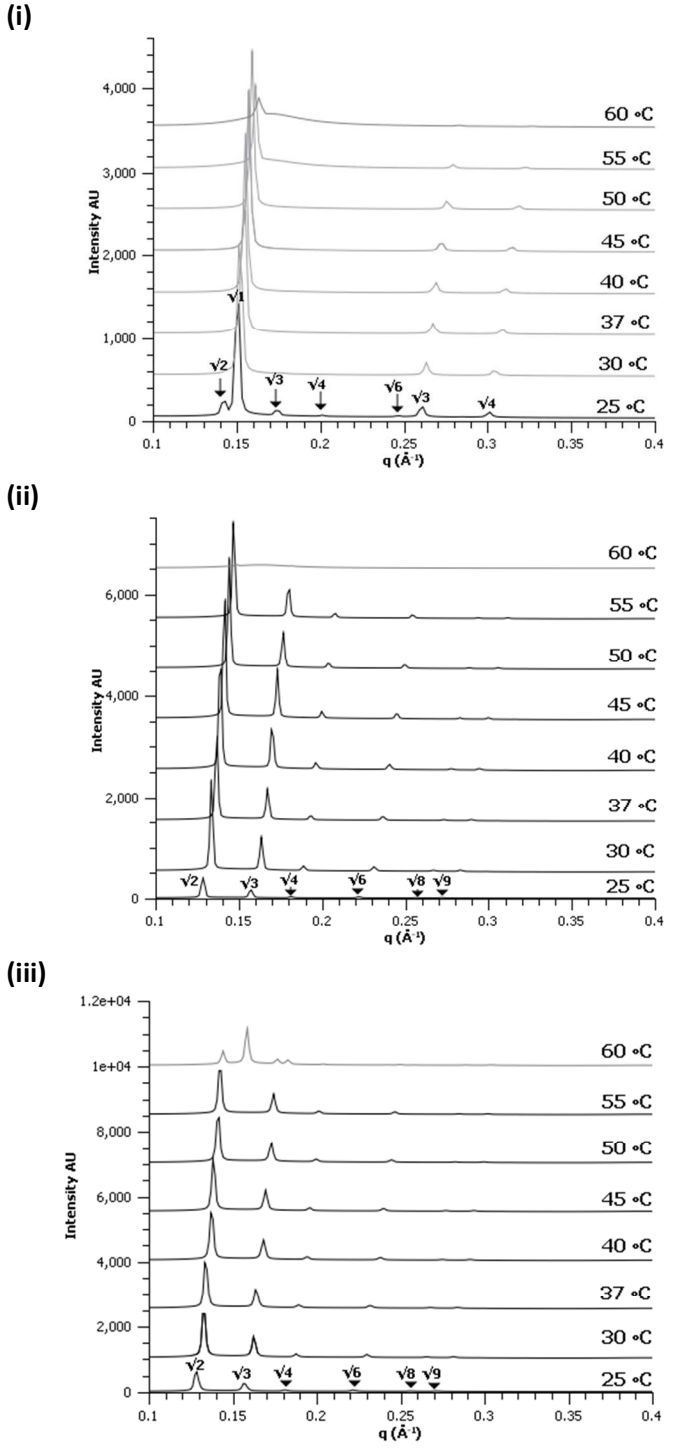

Figure 4. Stacked SAXS diffraction pattern, at 25 to 60 °C, for phytantriol dispersions stabilised with 1 wt. % of (i) Non-reduced $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$ copolymer, (ii) Reduced $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$ copolymer and (iii) F127 (control stabiliser). Arrows indicate Q_2^D cubic phase.

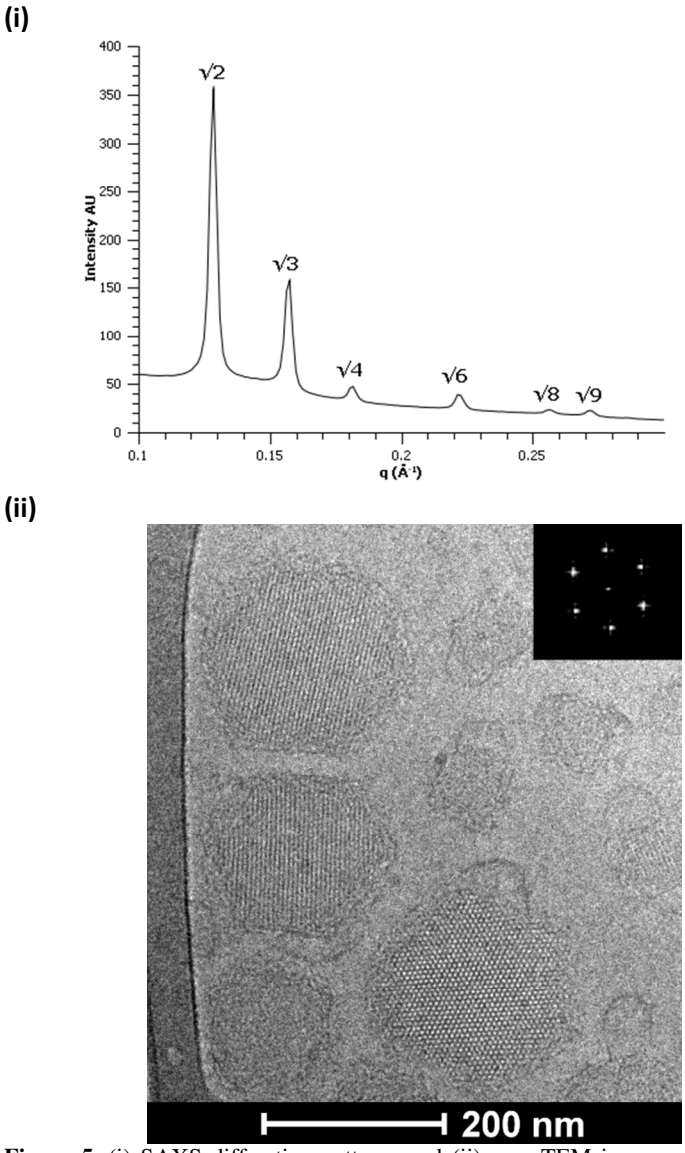

Figure 5. (i) SAXS diffraction patterns and (ii) cryo-TEM images for phytantriol dispersion stabilised with reduced $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$ copolymer at 25 °C using mol equivalent stabiliser concentration to F127 at 1.5 wt. %

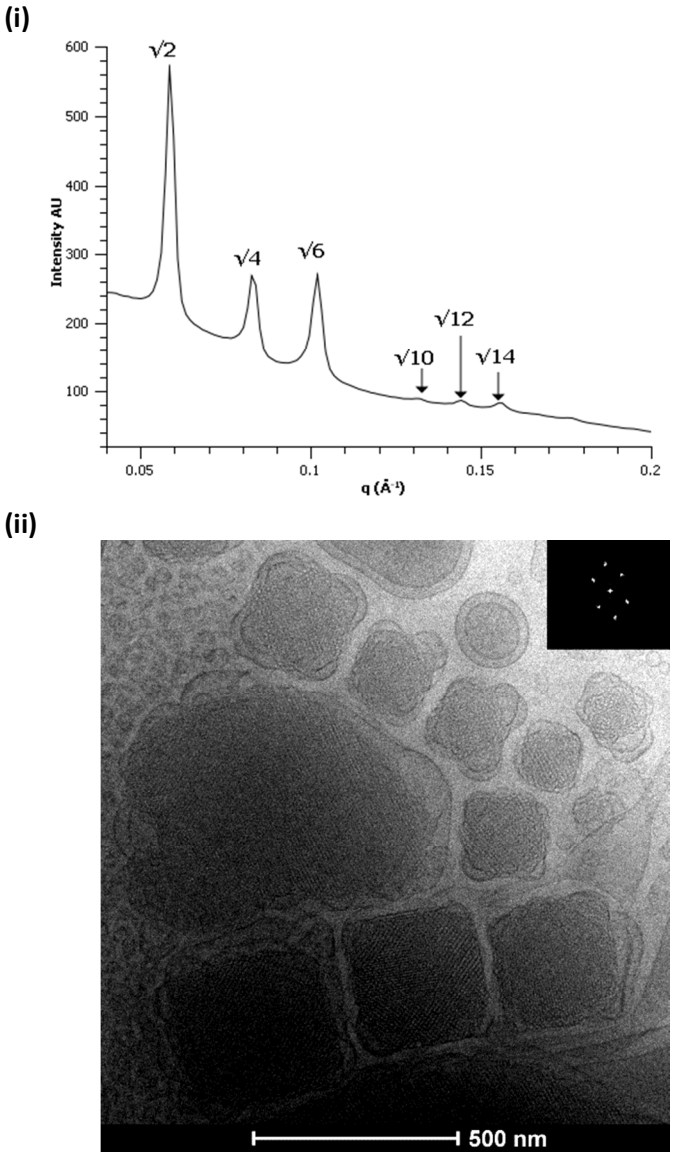

Figure 6. (i) SAXS diffraction patterns and (ii) cryo-TEM images for monoolein dispersion stabilised with reduced $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$ copolymer at 25 °C using mol equivalent stabiliser concentration to F127 at 1.5 wt. %

The reduced P(ODA)-*b*-P(PEGA-OMe) brush copolymers also stabilised monoolein dispersions at various stabiliser concentrations (i.e., mol equivalent concentrations to F127: 0.7, 1, 1.2, 1.5 and 2 wt. %) with the internal primitive Q_2^P cubic phase (Figure 6 and 7). The Q_2^P cubic phase did not change phase as the temperature was increased up to 66 °C while taking SAXS profiles (Figure 7). This is similar to F127 stabilised monoolein dispersions, which also resulted in a Q_2^P cubic phase, which did not change phase with increasing temperatures up to 66 °C (Figure 7). The Q_2^P monoolein cubosomes stabilised with 1 wt. % of $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$ had an average lattice parameter of 149.8 Å at 25 °C, which decreased as the temperature was increased. This result is comparable with literature reports of GMO systems stabilised by F127 at the same concentration (e.g., 130-140 Å at 25 °C).[12, 14, 17, 19, 25]

Cubosomes as well as vesicular structures were observed under cryo-TEM. Although cubosomes of different sizes existed, the majority of the nanostructured particles were approximately 200 nm in diameter as seen in Figure 5 and 6. The Fourier transform of the internal structure of the particle (Figure 5ii insert) shows a hexagonal arrangement with interplanar distances of about 6 nm. The internal structure is observed along the [111] axis, and the crystallographic planes observed are of the (110) type. This is compatible with the cubic structure of Pn3m symmetry with a lattice size of 8.5 nm.

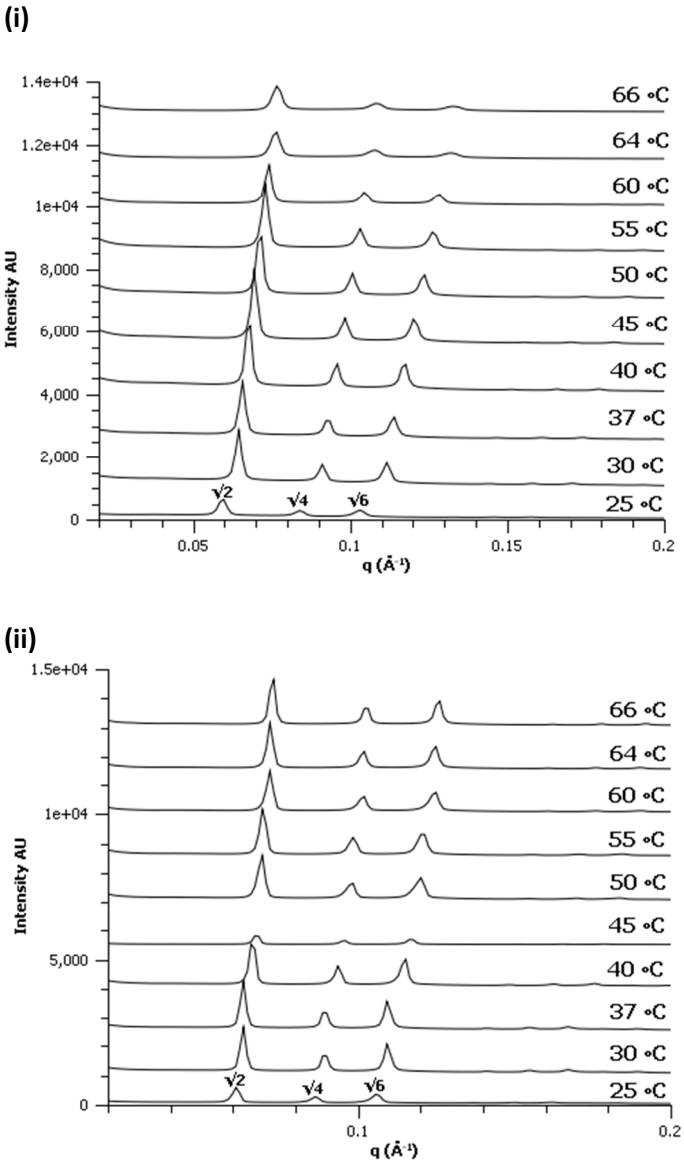

Figure 7. Stacked SAXS diffraction patterns, at 25 to 66 °C, for monoolein dispersions stabilised with 10 wt. % of (i) Reduced $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$ copolymer or (ii) F127 (control stabiliser)

Discussion

The structure of the copolymer used for steric stabilisation of lyotropic liquid crystalline bicontinuous cubic nanostructured particles is important. Although linear block copolymers (e.g., F127) have been commonly/traditionally used as steric stabilisers for cubosomes, this study has shown that more complex polymer designs, such as amphiphilic brush copolymers, can also be a viable alternative structural option for sterically stabilising cubosome dispersions. There are two trends that are prevalent from previous studies[17, 18, 25, 28] for improving the effectiveness of a steric stabiliser, which are gained by altering the structure of the stabiliser and these are: (1) increasing the hydrophilic block length (i.e., increasing the PEG length) and/or (2) increasing the number of hydrophilic blocks (i.e., increasing the number of PEG blocks in the copolymer structure).

The first trend can be seen in the Myrj®, Pluronic® and PEGylated-phytanyl copolymer stabiliser series, whereby increasing the length of the PEG domain on the stabiliser created greater colloidal stability for cubosome dispersions.[18, 28] For example, copolymers with 150 PEG units (i.e., Myrj®: PEG-150-stearate) or 132 PEG unit (i.e., Pluronic® F108) were found to be more effective stabilisers than their corresponding copolymers with only 100 PEG units on average (i.e., Myrj® 59 and Pluronic® F127).[18] In agreement with this trend it was observed in this study that increasing the hydrophilic PEGA-OMe block length (e.g., from 23 to 34 PEGA-OMe units) of the brush copolymer stabilisers, also resulted in more effective stabilisation of both phytantriol and monoolein cubosome dispersions. Although the PEG length within the PEGA-OMe 'brush-arms' remained the same (i.e., 9 PEG units on average), increasing the units of PEGA-OMe in the hydrophilic block increases the overall hydrophilicity of the copolymer causing a similar effect to increasing the PEG length of a stabiliser as seen in previous studies[17, 18, 25, 28].

The latter trend of improving the effectiveness of the steric stabiliser by increasing the number of hydrophilic domains in its amphiphilic structure can also be seen when comparing stabilisers from the Myrj® and Pluronic® series (i.e., Myrj® 59 and Pluronic® F127, which both have 100 PEG units on average). It was found that doubling the number of PEG domains in a steric stabiliser from a linear diblock copolymer (Myrj® 59) with one PEG block, to a triblock copolymer (Pluronic® F127) with two PEG blocks, resulted in improved effectiveness of the steric stabiliser at maintaining colloidal cubosome dispersions.[18] In a similar notion, increasing the PEG density in the structure of a stabiliser by using a brush configuration, such as the P(ODA)-*b*-P(PEGA-OMe) brush copolymers used in this study, has also shown to improve steric stabilisation effectiveness, especially over the triblock copolymer, F127. The improved steric stabiliser effectiveness produced by increasing the quantity/density of PEG arms in the hydrophilic PEGA-OMe brush structure is most likely due to it having a greater surface area-to-volume ratio than a linear copolymer structure, consequently allowing there to be increased steric hindrance coverage for the nanostructured particle.

The length of the hydrophobic domain of the P(ODA)-*b*-P(PEGA-OMe) brush copolymers was also found to influence its effectiveness as a steric stabiliser. When comparing the stabilisation of cubosome dispersions using brush copolymers with a similar hydrophilic domain size but different hydrophobic domain sizes (e.g., $P(ODA)_6$-*b*-$P(PEGA\text{-}OMe)_{27}$ vs. $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{23}$), it was found that copolymers with a longer hydrophobic domain (i.e., 10 ODA units) were more effective steric stabilisers. Similarly, it has previously been reported that increasing the length of the hydrophobic domain in PEGylated-phytanyl steric stabilisers also results in improved steric stabilisation of cubosome dispersions.[28] Improved stabilisation due to larger hydrophobic domains is likely caused by the stronger affinity created between the stabiliser and the nanostructured particle after increasing the hydrophobicity of the stabiliser.

Although these custom P(ODA)-*b*-P(PEGA-OMe) brush copolymers were synthesised with intent to optimally sterically stabilise monoolein and phytantriol cubosome dispersions, their brush copolymer structure has to our knowledge never been reported for stabilising lyotropic liquid crystalline nanostructured particles. However, this study validates the use and effectiveness of an amphiphilic brush copolymer structure for sterically stabilising

cubosome dispersions of different lipid compositions (i.e., both monoolien and phytantriol). Cubosome dispersions stabilised using P(ODA)-*b*-P(PEGA-OMe) brush copolymers were found to have similar phase behaviours to those stabilised with F127. Although the brush copolymer structure is different to that of triblock copolymer (i.e., Pluronic® F127), its ability to sterically stabilise cubosome dispersions could be attributed to the ratio of the amphiphilic domains; with a longer hydrophilic block than hydrophobic block, and a hydrophilic lipophilic balance (HLB) value greater or equal to 17, being favourable characteristics.

This study has also affirmed the possibility of polymerising novel custom steric stabilisers for cubosomes using RAFT polymerisation. However, this polymerisation technique results in the presence of a hydrophobic RAFT end-group. Results have shown that it is important to use the reduced form of the RAFT polymerised stabilisers for achieving optimal stability of phytantriol and/or monoolein cubosome dispersions because the presence of a dodecyl trithiocarbonate RAFT end-group, located on the terminal end of the hydrophilic block, significantly decreases the effectiveness of the P(ODA)-*b*-P(PEGA-OMe) brush copolymers as steric stabilisers. It is likely that due to the hydrophobic nature of the end-group the non-reduced copolymer acted like a triblock copolymer with hydrophobic end blocks, instead of its intended amphiphilic diblock brush copolymer structure. As reported in a previous study the position of the hydrophilic and hydrophobic blocks in an amphiphilic triblock copolymer used as a steric stabiliser is important.[18] Only triblock copolymers with hydrophilic end blocks are effective stabilisers as structures with hydrophobic ends have the tendency to promote aggregation via particle bridging.

In addition to discovering a novel steric stabiliser structure, this study allows us to pursue/view custom steric stabilisers as viable options for stabilising lyotropic liquid crystalline nanostructured particles (e.g., cubosomes) for drug delivery systems. Advantages of customising the design of steric stabilisers include the ability to optimise the structure and block lengths for stabilising dispersions for different lipids and also allow options for functionalisation. Functionalisation of the steric stabiliser, specifically attaching a functional/targeting moiety (e.g., antibody fragment) to the terminal end of the hydrophilic domain, would consequently allow site specific targeting (e.g., tumour sites) of the nanostructured particle and thus enable active drug delivery of these systems to be explored. This would also have broader implications and applications of these systems for MRI imaging and drug delivery applications, as these systems are versatile and multifaceted with the capacity to contain either hydrophilic or hydrophobic therapeutics, imaging/contrast agents[45] and other nano-structures (e.g., gold nanorods)[46] that can act as switches for initiating controlled drug release. With the combination of specific site drug delivery and locating its position in the body and also controlling the drug release, the potential for more effective drug treatments with less adverse side-effects is possible.

Conclusion

Poly(octadecyl acrylate)–*block*-poly(polyethylene glycol methyl ether acrylate) amphiphilic brush copolymers are efficient novel steric stabilisers for lyotropic liquid crystalline nanostructured colloidal particles. When comparing non-reduced and reduced P(ODA)-*b*-P(PEGA-OMe) brush copolymers for their steric stabiliser effectiveness, it was found that reduced brush copolymers, provided significantly better steric stabilisation without the dodecyl trithiocarbonate RAFT end-group. However, the stabilisation of cubosomes using the non-reduced P(ODA)-*b*-P(PEGA-OMe) brush copolymers, demonstrates potential for custom brush copolymer stabilisers to functionalise the RAFT copolymer end-group for active targeting of the cubosome, whilst maintaining steric stabilisation.

The most effective steric stabiliser of the P(ODA)-*b*-P(PEGA-OMe) brush copolymer series, for both phytantriol and monoolein cubosome dispersions, consisted of the longest hydrophilic and hydrophobic brush blocks, $P(ODA)_{10}$-*b*-$P(PEGA\text{-}OMe)_{34}$. However, all six reduced P(ODA)-*b*-P(PEGA-OMe) brush copolymers were able to sterically stabilise monoolein and phytantriol dispersions, with either equivalent or better steric stabiliser effectiveness to the standard control stabiliser, Pluronic® F127. This demonstrates the potential of exploring new custom polymers and/or different copolymer structures for improving the steric stabilisation of lyotropic liquid crystalline nanostructured particles. Furthermore, this study initiates the opportunity to develop novel custom functionalised steric stabilisers for exploring active targeting in these lyotropic liquid crystalline nanostructured particle systems, for drug delivery and MRI imaging applications.

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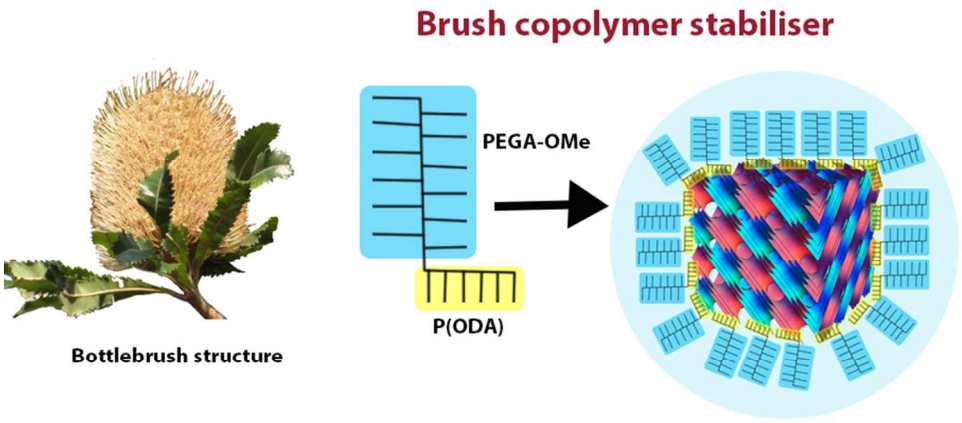

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