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Novel Steric Stabilizers for Lyotropic Liquid Crystalline Nanoparticles: PEGylated-Phytanyl Copolymers

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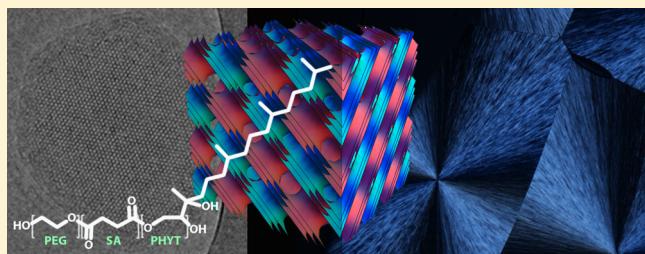
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Supporting Information

ABSTRACT: Lyotropic liquid crystalline nanostructured particles (e.g., cubosomes and hexosomes) are being investigated as delivery systems for therapeutics in biomedical and pharmaceutical applications. Long term stability of these particulate dispersions is generally provided by steric stabilizers, typically commercially available amphiphilic copolymers such as Pluronic F127. Few examples exist of tailored molecular materials designed for lyotropic liquid crystalline nanostructured particle stabilization. A library of PEGylated-phytantriol copolymers (PEG-PHYT) with varying PEG

molecular weights (200–14K Da) was synthesized to assess their performance as steric stabilizers for cubosomes and to establish structure-property relationships. The PEGylated-lipid copolymers were first found to self-assemble in excess water in the absence of cubosomes and also displayed thermotropic liquid crystal phase behavior under cross-polarized light microscopy. An accelerated stability assay was used to assess the performance of the copolymers, compared to Pluronic F127, for stabilizing phytantriol-based cubosomes. Several of the PEGylated-lipid copolymers showed steric stabilizer effectiveness comparable to Pluronic F127. Using synchrotron small-angle X-ray scattering and cryo-transmission electron microscopy, the copolymers were shown to retain the native internal lyotropic liquid crystalline structure, double diamond cubic phase (Q_2^D), of phytantriol dispersions; an important attribute for controlling downstream performance.



1. INTRODUCTION

Inverse bicontinuous cubic and hexagonal lyotropic liquid crystalline dispersions of amphiphiles (i.e., cubosomes and hexosomes, respectively) are of interest in drug delivery applications due to their compartmentalized ordered internal structure, high lipid content, and large surface area. Lipids, such as monolein (GMO) and phytantriol, are common examples of amphiphile building blocks for lyotropic liquid crystalline particles.^{1–3} Their amphiphilic self-assembly feature enables them to be compatible with both lipophilic and hydrophilic therapeutics or biomedical imaging agents.^{4–7} Cubosome and hexosome dispersions are typically only colloidally stable for extended periods when in the presence of a steric stabilizer which prevents particle aggregation. The range of steric stabilizers that can successfully disperse lyotropic liquid crystalline particles remains limited.^{8–10}

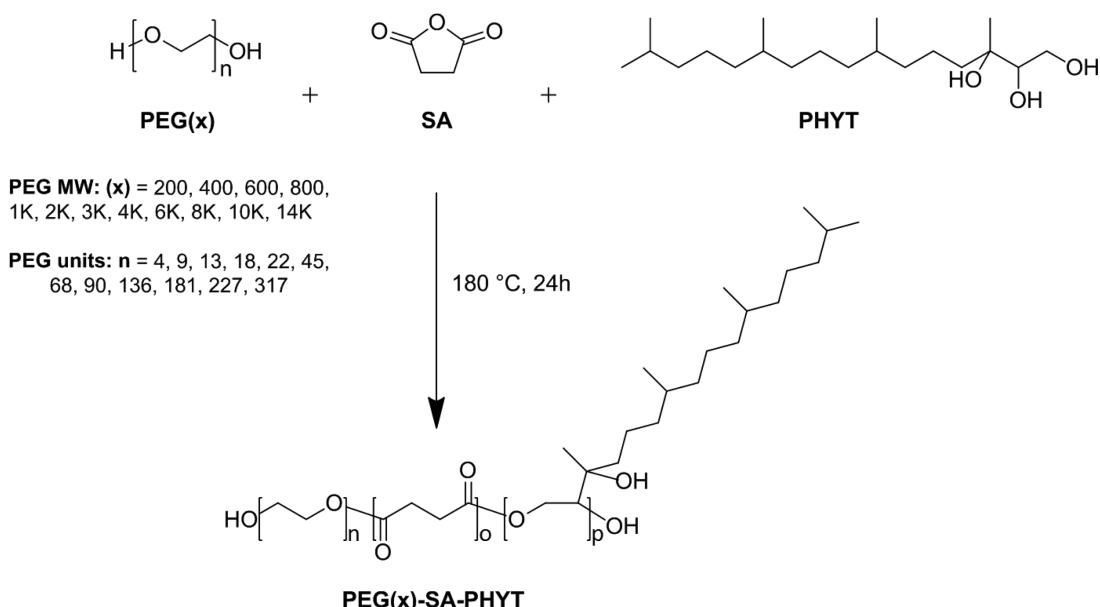
The current gold standard in lyotropic liquid crystalline particle stabilization is Pluronic F127 (F127). F127 is a

poly(ethylene glycol)–poly(propylene oxide)–poly(ethylene glycol) nonionic triblock copolymer (PEG-PPO-PEG), with an average molecular weight of 12 600 and approximately 100 PEG units on average on both sides of 65 PPO units on average.^{11–16} Although popular, F127 has been shown to have restricted stabilizer effectiveness for the long term stability of cubosomes.¹⁷ Alternative steric stabilizers that have been investigated for cubosomes include Pluronic F108,¹⁶ β -casein,¹⁸ Myrij 59,⁸ Laponite,¹⁹ modified cellulose,²⁰ ethoxylated phytosterol,²¹ Polysorbate 80,⁸ and silica particles.²² For amphiphilic stabilizers (e.g., F127), the lipophilic domain (e.g., PPO) anchors to the lipid bilayer of the lyotropic liquid crystalline system. The stabilizer thus has a strong affinity to the cubosome, and this affinity can be tuned by altering lipid-to-

Received: April 17, 2014

Revised: July 20, 2014

Scheme 1. Synthesis Scheme of the Copolymers Made of Polyethylene Glycol (PEG), Phytantriol (PHYT), and Succinic Anhydride (SA) by Polycondensation^a



^aPEG MW is denoted with (x), while the number of PEG units, SA units, and PHYT units on average are denoted by *n*, *o*, and *p*, respectively. Polyethylene glycol units were calculated in accordance to $C_{2n}H_{4n+2}O_{n+1}$.

stabilizer compatibility. The hydrophilic domain remains in the water or polar region of the self-assembled structures. Stabilizers have been found to associate with both the internal and external surfaces of the cubosomes.¹⁷ The hydrophilic domain often consists of PEG. This chemical moiety has been found to provide stealth *in vivo*,²³ thus prolonging the circulation time of nanoparticles *in vivo*.^{24–28}

Stabilizers can have an effect on the internal nanostructure of the dispersed lyotropic liquid crystalline particles. Studies using GMO-based cubosomes stabilized by F127 have shown that a transition between two cubic phases can be induced by the lipophilic domain (e.g., PPO) of the stabilizer being embedded in the cubosome bilayer. This drives a phase transition from the native double diamond phase to a primitive phase ($Q_2^D(P_{n3m})$ to $Q_2^P(I_{m3m})$). In contrast, F127 adsorbs at the interface of phytantriol-based cubosomes, with PPO occupying a finite interfacial area, limiting the available surface area for further stabilizer adsorption.¹⁷ The association of the lipophile to the cubosome was found to be strong and irreversible as the stabilizer did not desorb from the cubosome after dilution of the system.¹⁷ An effective steric stabilizer will therefore require a lipophile with a strong lipophilic affinity, while preserving the cubic internal nanostructure of the lyotropic liquid crystalline phase to ensure stability of the dispersion.

The limited chemical space explored, with respect to the nature of the steric stabilizers, can be attributed to the time-consuming nature of single sample preparation and material characterization techniques, impeding the progress of steric stabilizer screening. The development of high-throughput methodologies has recently enabled the implementation of rapid preparation and screening protocols^{8,16,29,30} In previous work, a number of commercially available polymers were examined as potential steric stabilizers for cubosomes. The Pluronic polymer series¹⁶ and later the Myrj polymer series⁸ were screened as stabilizers for phytantriol and monoolein cubosomes. Studies using the Pluronic polymer series found

that changes to the internal structure of the dispersions were directly linked to the internalization of the stabilizer within the lipidic structure. Eliminating or reducing the internalization of the stabilizer into the dispersed particles reduced the propensity for changes to the internal nanostructure. This was illustrated by the conservation of the double diamond cubic phase (Q_2^D) for monoolein dispersions when using Pluronic F108, which has the longest PEG chain of the Pluronics commercially available. It was suggested that stabilizers with longer PEG chains (i.e. >100 PEG units on average) had increased presence on the surface of the particle, which alludes to the desire to investigate molecular structures with larger, controllable PEG content, such as custom synthesized copolymers. Pluronics are also difficult to functionalize where further functionality such as targeting by ligands to specific cell types is required. Custom copolymers for cubosomes are therefore attractive from both a colloidal stability and functionalization perspective.

PEGylated-lipid copolymers have been reported for use in self-assembled drug delivery systems (e.g., micelle drug delivery systems).^{31–33} Rouxhet et al. synthesized AB type PEGylated-lipid copolymers, with the lipid component consisting of monoglyceride.³³ These amphiphilic PEGylated-lipid copolymers were observed to self-assemble into micellar systems, as well as solubilize poorly water-soluble drugs. Such amphiphilic polymers with lipid-based hydrophobic domains could provide a suitable surface “anchor” in lipid membranes and could therefore constitute a new class of steric stabilizers for cubosomes. Consequently, in the current study, a series of PEGylated-lipid copolymer steric stabilizers incorporating a common lyotropic liquid crystal lipid, phytantriol, as the lipophilic component of the copolymers, was synthesized and characterized for their self-assembly behavior and stabilization of cubosomes. The polycondensation synthesis employed (Scheme 1) was adapted from Rouxhet et al.³³ because it was an expedient way to develop some structure-performance relationships. Although there may be more controlled polymer-

ization approaches reported,^{34–37} the Rouxhet et al.³³ approach was ultimately selected for synthetic simplicity and for amenity to a combinatorial chemistry approach. The novel PEGylated-lipid copolymer steric stabilizers were developed to contain a series of various PEG lengths (i.e. from 200 to 14K) and, similarly to Rouxhet et al., various PEG to lipid ratios. The incremental variation of the steric stabilizer series allowed a comprehensive assessment of the structure–property relationship for the effectiveness of steric stabilization of lyotropic liquid crystalline nanostructured particles.

2. MATERIALS AND METHODOLOGY

2.1. Materials. Phytantriol (3,7,11,15-tetramethylhexadecane-1,2,3-triol) was a gift from DSM Nutritional Products, Wagga Wagga, NSW, Australia. Polyethylene glycol 200 and 400 were purchased from BDH Laboratory Reagents, Poole, U.K. Polyethylene glycol 4000 was purchased from BDH Chemicals Australia, Port Fairy, VIC, Australia. Polyethylene glycol 600, 2000, 8000, 14 000, succinic anhydride 99%, 0.01 M phosphate buffered saline solution (pH 7.4), fluorescein sodium salt, and Pluronic F127 were purchased from Sigma-Aldrich, Sydney, NSW, Australia. Polyethylene glycol 800, 1000, and 6000 were purchased from ICI Australia Operations Pty Ltd, Melbourne, VIC, Australia. Polyethylene glycol 3000 and 10 000 were purchased from Merck-Schuchardt, Hohenbrunn, Germany.

2.2. Methodology. **2.2.1. Polymer Synthesis and Characterization.** The copolymers were synthesized by polycondensation according to the synthesis pathway reported in Scheme 1.³³ Briefly phytantriol, succinic anhydride, and polyethylene glycol were placed under nitrogen and the temperature raised to 180 °C. The reaction was maintained at 180 °C for 24 h. Different mole percent ratios for developing five different copolymer series were made using a range of polyethylene glycol with molecular weights (MW) of 200, 400, 600, 800, 1000 (1K), 2000 (2K), 3000 (3K), 4000 (4K), 6000 (6K), 8000 (8K), 10 000 (10K), and 14 000 (14K). A total of 60 copolymers were synthesized in this study.

The nomenclature adopted for the copolymers assumed a 50 mol % succinic anhydride (SA) content (based on the mole ratio of reactants), and indicates the PEG length and the PEG to phytantriol ratio. These polymers are indicated as PEG(x)_yPHYT_z, where x denotes the PEG length, y denotes the mole fraction of PEG in the polymer, and z denotes the mole fraction of phytantriol. Thus, because the SA content is always 50 mol %, $y + z = 50$ mol % for all polymers.

The polymer composition and residual monomer content were analyzed by NMR. The copolymers were dissolved in deuterated chloroform and spectra for structural assignments were obtained with a Bruker Avance 400 MHz spectrometer (¹H 400 MHz, ¹³C 100 MHz).

Molecular weights of the polymers were determined by gel permeation chromatography (GPC) performed in chloroform (1.0 mL/min) at 30 °C using a Waters 2695 separations module, with a Waters 2414 refractive index detector and a Waters 2996 photodiode array detector, a series of four Polymer Laboratories PLGel columns (3 × 5 μm Mixed-C and 1 × 3 μm Mixed-E), and Empower Pro Software. GPC was calibrated with narrow polydispersity polystyrene standards (Polymer Laboratories EasiCal, M_w from 264 to 256 000), and molecular weights are reported as polystyrene equivalents based on the refractive index detector.

2.2.2. Polymer Self-Assembling Properties. The critical aggregation concentration (CAC) of the copolymers was determined by using changes in the scattered light intensity from dynamic light scattering (DLS). DLS was performed using a DynaPro plate reader (Wyatt Technology, Santa Barbara, CA). The DLS instrument uses a 50 mW programmable laser ($\lambda = 831.5$ nm) with a detection at 158° and a thermostated sample chamber set to 25 °C. The viscosity and refractive index of water at 25 °C, 0.8937 cP and 1.333, respectively, were used for all measurements. In the absence of aggregates the intensity of backscattered light is comparable to that of the solvent. In the presence of aggregates, the intensity of backscattered light

increases with increasing concentration of aggregates.³⁸ Solutions were prepared in double distilled water by dilution from 20 mg/mL stock solutions of the steric stabilizers and analyzed using a polystyrene clear bottom low volume 384 well plate (product #3540, Corning). Three repeats were made per sample. Ten acquisitions were collected for each sample to ensure reproducibility. Water wells were used as a blank. Intensity and size information was obtained from the Wyatt DynaPro plate reader using the software package DYNAMICS v.7. CAC measurements were averaged from the three repeat measurements.

Assessment of lyotropic phase behavior of bulk copolymer in excess water was determined by using cross-polarized light microscopy (CPLM). Briefly, copolymer was melted onto a glass slide, covered with a coverslip, and flooded with water for observation under a Nikon Eclipse 80i microscope (Nikon Corporation, Japan), with ×10 magnification, to obtain water penetration scans at temperatures 20–65 °C. Anisotropic phase behavior was determined using cross-polarizers, to detect a birefringent appearance of the sample.³⁹ Further assessment of lyotropic liquid crystalline phases occurring in the self-assembly of the copolymers in water was determined by using high throughput small-angle X-ray scattering (SAXS), for two different systems: (i) with excess polymer (>60% polymer content) and (ii) with excess water (>60% hydration).

2.2.3. Preparation of Nanostructured Particles. Lyotropic liquid crystalline dispersions were formed at a concentration of 100 mg/mL of phytantriol in 500 μL of 0.01 M phosphate buffered saline at pH 7.4 (PBS), with 1 wt % (i.e. 10 mg/mL) of each PEG-PHYT copolymer as the steric stabilizer. The copolymers with PEG MW ≤ 1000 were dissolved in chloroform, containing 50 mg of phytantriol. These samples were then left in a vacuum desiccator over 14 days to ensure solvent removal. PBS (500 μL) was then added to each sample. Copolymers with PEG MW > 1000 were dispersed in 500 μL of PBS. Once completely dispersed, phytantriol (50 mg) was added to each sample. Following combination of lipid, stabilizer, and water, each sample was sonicated using a Misonix ultrasonic liquid processor microtip probe sonicator (Misonix Inc., NY), with a 418 Misonix probe. The sequence programmed for the sonication of samples consisted of three programs, which were implemented in succession without any delay time. Program 1 settings: 50 Amplitude, 30s Process time, 3s Pulse-time On, 2s Pulse-time Off. Program 2 settings: 45 Amplitude, 1 min Process time, 2s Pulse-time On, 4s Pulse-time Off. Program 3 settings: 40 Amplitude, 1 min Process time, 2s Pulse-time On, 4s Pulse-time Off. The sequence resulted in a total sonication time of 2.5 min per sample. The sample temperature during sonication was monitored and observed to be between 65 to 70 °C.

2.2.4. Characterization of Colloidal Stability, Internal Structure, and Particle Morphology. The copolymers were assessed for performance as stabilizers for lyotropic liquid crystal dispersions using a visual assessment and an accelerated stability assay. An accelerated stability assay was developed to quantify the steric stabilizer effectiveness between fair to excellent lyotropic liquid crystal stabilizers that passed the initial visual assessment of particle stability.⁴⁰ Briefly, lyotropic liquid crystal phytantriol dispersions were mixed at equal volumes with hydrophilic dye solution, fluorescein sodium salt solution (3.1×10^{-4} mg/mL) (i.e. 15 μL cubosome sample mixed with 15 μL dye solution) and pipetted into a 384 black round well Corning microplate. Control samples were prepared using 15 μL of PBS and 15 μL of dye solution. The same was done using PBS buffer solution instead of dye solution for control samples. Three repeats were made for each sample. Fluorescence signal intensities were taken pre- and postcentrifugation. The centrifugation of plates was performed with a Heraeus Multifuge ×3 centrifuge (Thermo Scientific, Germany). Fluorescence signal measurements, with emission at 530 nm and excitation at 480 nm, were taken using a FlexStation3Multimode microplate reader (Molecular Devices Company) and processed on SoftMax Pro software. The plate was initially spun at 645g (RCF) or 1800 rpm for 5 min, measured for fluorescence signal, and then respun at 796g (RCF) or 2000 rpm for 5 min. Dispersions stabilized with F127 at 0.3, 0.5, 0.7, 1, and 1.2 wt % were used as a comparison for the ASA, where wt % is relative to total dispersion.

Table 1. PEG-PHYT Copolymer Molecular Weights (M_w , M_n), Dispersity (D), Physical State at Room Temperature 25 °C (S), Hydrophilic–Lipophilic Balance (HLB), and Critical Aggregation Concentration (CAC)

polymer composition (mol %)	PEG(x)	M_w^a	M_n^a	D^a	S ^b	HLB ^c	CAC (μM) ^d	ΔG_{agg} (kJ mol ⁻¹) ^e
PEG(x) ₁₀ PHYT ₄₀	PEG200	1629	1358	1.3	L	6.9		
	PEG400	1259	914	1.4	L	8.1		
	PEG600	1259	901	1.4	L	9.1		
	PEG800	1278	869	1.5	L	9.9		
	PEG1K	1812	1014	1.8	W/G	10.6		
	PEG2K	5351	4965	1.1	W/S	13.1	1.6	-33.1
	PEG3K	5870	2150	2.7	W/G	14.5	0.02	-44.3
	PEG4K	8089	7353	1.1	W/G	15.5	1	-34.2
	PEG6K	13 721	11 442	1.2	S	16.6	0.2	-38.2
	PEG8K	20 379	17 886	1.1	S	17.3	0.05	-41.9
PEG(x) ₂₀ PHYT ₃₀	PEG10K	16 585	11 414	1.5	S	17.8	0.06	-41.4
	PEG14K	28 775	23 985	1.2	S	18.3	0.1	-40.0
	PEG200	1700	1385	1.2	L	9.5		
	PEG400	2005	1466	1.4	L	11.3		
	PEG600	1644	1118	1.5	L	12.6		
	PEG800	2354	1406	1.7	L	13.6		
	PEG1K	3195	1696	1.9	W/G	14.3		
	PEG2K	5437	4425	1.2	W/S	16.4	1.6	-33.1
	PEG3K	8054	6283	1.3	W/G	17.4	1	-34.2
	PEG4K	9345	7193	1.3	W/G	17.9	2.7	-31.8
PEG(x) ₂₅ PHYT ₂₅	PEG6K	12 430	9219	1.3	S	18.5	2	-32.5
	PEG8K	38 805	33 867	1.1	S	18.9	0.7	-35.3
	PEG10K	13 307	9171	1.5	S	19.1	1.9	-32.7
	PEG14K	27 782	21 954	1.3	S	19.3	0.9	-34.5
	PEG200	1660	1366	1.2	L	11.0		
	PEG400	1641	1086	1.5	L	12.9		
	PEG600	1580	993	1.6	L	14.2		
	PEG800	2184	1304	1.7	L	15.0		
	PEG1K	3124	1646	1.9	W/G	15.7		
PEG(x) ₃₀ PHYT ₂₀	PEG2K	5356	4510	1.2	W/S	17.4	0.5	-35.9
	PEG3K	8687	7325	1.2	W/S	18.1	1	-34.3
	PEG4K	6312	2444	2.6	W/G	18.5	12	-28.1
	PEG6K	13 395	10 717	1.2	S	19.0	1.9	-32.7
	PEG8K	10 422	3870	2.7	S	19.2	2.5	-32.0
	PEG10K	18 852	13 884	1.4	S	19.4	1.4	-33.4
	PEG14K	25 429	13 969	1.8	S	19.5	3	-31.5
	PEG200	2898	2301	1.3	L	12.5		
	PEG400	2074	1483	1.4	L	14.4		
	PEG600	1800	1171	1.5	L	15.5		
PEG(x) ₄₀ PHYT ₁₀	PEG800	2362	1334	1.8	L	16.3		
	PEG1K	3970	2459	1.6	W/G	16.8		
	PEG2K	5182	4274	1.2	W/S	18.2	0.5	-35.8
	PEG3K	6761	5935	1.1	S	18.7	3.8	-30.9
	PEG4K	7907	7243	1.1	S	19.0	9.8	-28.6
	PEG6K	13 602	11 132	1.2	S	19.3	1.9	-32.7
	PEG8K	14 594	7765	1.9	S	19.5	5.3	-30.1
	PEG10K	19 276	14 231	1.4	S	19.6	4	-30.8
	PEG14K	27 161	20 671	1.3	S	19.7	2.9	-31.6
	PEG200	1717	1324	1.3	L	15.9		
	PEG400	1895	1195	1.6	L	17.3		
	PEG600	2148	1310	1.6	L	18.0		
	PEG800	2885	1548	1.9	L	18.4		
	PEG1K	4820	2246	2.0	W/G	18.6		
	PEG2K	5109	4327	1.2	W/S	19.3	0.2	-38.5
	PEG3K	16 254	12 261	1.3	S	19.5	4.8	-30.4
	PEG4K	9600	7877	1.2	S	19.6	8	-29.1

Table 1. continued

polymer composition (mol %)	PEG(<i>x</i>)	<i>M</i> _w ^a	<i>M</i> _n ^a	<i>D</i> ^a	<i>S</i> ^b	HLB ^c	CAC (μM) ^d	ΔG_{agg} (kJ mol ⁻¹) ^e
	PEG6K	12 198	8977	1.4	S	19.7	6.4	-29.6
	PEG8K	20 426	18 107	1.1	S	19.8	12	-28.1
	PEG10K	22 290	17 483	1.3	S	19.8	10	-28.5
	PEG14K	28 529	23 227	1.2	S	19.9	8.2	-29.0

^aApparent weight-averaged molecular weight (*M*_w), number-average molecular weight (*M*_n), and dispersity (*D*) of the phytanyl-based PEGylated copolymers determined by chloroform gel permeation chromatography, with the molecular weights in polystyrene equivalents. Dispersity is calculated using *M*_w/*M*_n. ^bThe physical state of the copolymers at room temperature (25 °C): L, Liquid; W/G, Waxy/Gel; W/S, Waxy/Solid; S, Solid. ^cHLB calculated using Griffin's method for nonionic surfactants HLB = 20*M*_h/M, where *M*_h is the molecular mass of the hydrophilic portion of the molecule and M is the molecular mass of the whole molecule.⁶¹ ^dCAC: with micromole (μM) calculation using MW determined by chloroform gel permeation chromatography (GPC). ^eGibbs free energy of aggregation⁶² (ΔG_{agg}) calculated using $\Delta G_{\text{agg}} = RT \ln(\text{CAC})$, where R is the universal gas constant and T is the absolute temperature.

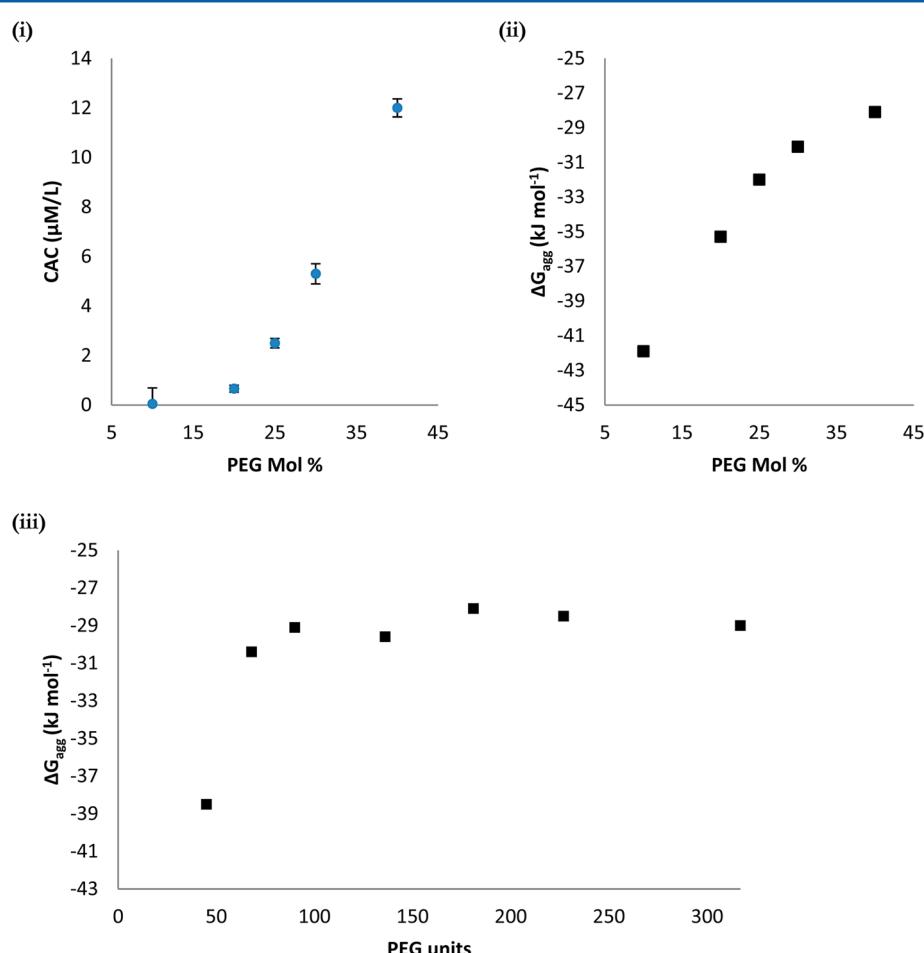


Figure 1. (i) CAC and (ii) ΔG_{agg} values for PEG8K_yPHYT_z, where *y* = 10–40 mol % and *z* = 10–40 mol %. (iii) ΔG_{agg} values vs PEG units for PEG(*x*)₄₀PHYT₁₀. Error bars represent the standard deviation.

The principle of this assay is that aggregation (that typically results in increased creaming) correlates with increased fluorescence signal intensities. Therefore, the magnitude of the change in fluorescence signal intensity following centrifugation is proportional to creaming levels. To differentiate steric stabilizer efficacies, comparing changes in fluorescence signal intensities is required. Good steric stabilizers are able to maintain a stable colloidal dispersion over time and therefore after centrifugation. Thus, the less creaming that occurs within a sample during the accelerated stability assay (i.e. minimal change in fluorescence signal intensity), the more stable the colloidal dispersion. This is indicative of the effectiveness of the steric stabilizer and can be quantifiably compared to a control system, such as F127.

Particle size and polydispersity of dispersed samples were determined by dynamic light scattering using a DynaPro plate reader (Wyatt Technology, Santa Barbara, CA). Particle size and poly-

dispersity from the DLS instrument were averaged from three repeat measurements. The viscosity of water was assumed, and the samples were run at 25 °C.

Dispersed samples visually assessed to be milky white with little to no aggregation, were further assessed for lyotropic liquid crystalline nanostructured particle phase behavior, using SAXS and cryo-TEM imaging. SAXS can be used to establish the phase structure (i.e. internal long-range order of the liquid crystal lattice) of the dispersed particle samples at selected temperatures. SAXS data was collected at the Australian Synchrotron using a beam with wavelength $\lambda = 1.033 \text{ \AA}$ and a typical flux of approximately 10^{13} photons/s. Two-dimensional diffraction patterns were recorded on a Dectris-Pilatus2 1-M detector. A silver behenate standard (*d*-spacing = 58.38 Å) was used for *q*-scale calibration. The samples were loaded into quartz glass 1.5 mm capillaries (Hampton Research) and positioned in a custom-designed

sample holder capable of holding 34 capillaries, and the temperature controlled to ± 1.0 °C between 20 and 75 °C.³⁰ Temperature control was via a recirculating water bath (Huber, Germany). SAXS was performed on dispersions from 25 to 65 °C at 5 °C increments. The exposure time for each sample was 1 s. SAXS data was analyzed using an IDL-based software package: AXcess.⁴¹

A laboratory-built humidity-controlled vitrification system was used to prepare the samples for cryo-TEM. Humidity was kept close to 80% for all experiments, and ambient temperature was 22 °C. These were the optimal conditions for sample preparation of lyotropic liquid crystalline nanostructured particle samples. Copper grids (200-mesh) coated with perforated carbon film (Lacey carbon film: ProSciTech, Kirwan, Qld, Australia) were glow discharged in nitrogen to render them hydrophilic. Aliquots of the sample (4 μ L) were pipetted onto each grid prior to plunging. After 30 s adsorption time, the grid was blotted manually using Whatman 541 filter paper, for 2 s. Blotting time was optimized 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) 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/ \AA^2 for all imaging. Images were recorded using a FEI Eagle 4k \times 4k CCD camera at magnifications in the range 15 000 \times to 40 000 \times .

3. RESULTS

A library of 60 PEGylated-lipid copolymers (PEG-PHYT) was synthesized using the polycondensation scheme (Scheme 1) adapted from Rouxhet et al.³³

Part I: Copolymers. **3.1. Polymer Characterization.** Polymers composed of 10–40 mol % PEG with molecular weights between 200 and 14K Da, 10–40 mol % phytantriol (PHYT) and all containing 50 mol % succinic anhydride (SA) were synthesized. The polycondensation of the different monomers, represented in Scheme 1, will lead to random copolymers. Weight-averaged molecular weight (M_w), number-average molecular weight (M_n), and dispersity ($M_w/M_n = D$) of the synthesized polymers were determined by GPC using chloroform as the eluent and are shown in Table 1. The dispersities were determined to be between 1.1 and 2.7, with most copolymers having $D < 1.5$.

3.2. Polymer Self-Assembling Properties. The self-assembly behavior of the polymers in excess water was characterized using dynamic light scattering to determine their critical aggregation concentration. Subsequently, the cross-polarized light microscopy technique was used to establish whether the polymers formed thermotropic liquid crystalline phases and whether lyotropic liquid crystals were formed in the presence of water.

3.2.1. Critical Aggregation Concentration (CAC) and Gibbs Free Energy of Aggregation (ΔG_{agg}). The critical aggregation concentration of each polymer, with a PEG moiety greater than 45 units on average (i.e. PEG2K), was established using dynamic light scattering as shown in Table 1. The scattered light intensity was measured over a serial dilution of the copolymers in water. Copolymers with PEG MW ≤ 1 K were excluded from the study due to their very low water solubility.

As expected, increasing the molar percentage (PEG mol %) of PEG units within the polymers typically resulted in a corresponding increase in CAC (Table 1). This trend was most pronounced across the 10, 20, 25, 30, and 40 PEG mol % copolymers with higher PEG molecular weights (i.e.,

\geq PEG6K). For example, the CACs for the PEG8K copolymers increased gradually with PEG molar ratio (Figure 1).

Increasing the PEG MW of the copolymer series while maintaining the same molar percentage of PEG to lipid generally resulted in an increase in the CAC. For example, the CAC generally increased for PEG2K (CAC value of 0.2 μ M) to PEG14K (CAC value of 8.2 μ M) in the 40 PEG mol % series (Table 1).

The Gibbs free energy of aggregation (ΔG_{agg}) in all cases is negative, confirming the spontaneity of aggregate formation and the spontaneity is generally observed to be much higher for soluble copolymers with a lower PEG mol % (i.e. 10 PEG mol %) (Table 1 and Figure 1).

3.2.2. Self-Assembly of Copolymers Using Cross-Polarized Light Microscopy (CPLM). Polymer self-assembly was examined using cross-polarized light microscopy to assess the thermotropic and lyotropic phase behavior properties. Temperature and penetration scans of PEG-PHYT copolymers with 10, 20, 25, 30, and 40 PEG mol %, where the PEG MW is 1K to 14K, were performed over a temperature range of 20–60 °C. The polymer series showed some anisotropic and isotropic phases, indicative of liquid crystalline properties (see the Supporting Information for data).³⁹

3.2.3. Lyotropic Liquid Crystalline Phase Behavior in Excess Water. Upon hydration in water, at either 25 or 30 °C, 23 of the 60 copolymers that were synthesized displayed an isotropic band near the water interface. The high viscosity upon shearing of this band suggested that it may be a cubic phase. The majority of copolymers that displayed cubic isotropic bands had a PEG molecular weight range between 1K and 14K and a PEG molar ratio typically between 10 and 30%.

One particular example, PEG10K₁₀PHYT₄₀, displayed an isotropic region, believed to be a cubic phase, above 35 °C, which slowly formed and expanded until the entire copolymer material dissolved, leaving only an aqueous solution visible on the microscope slide at 55 °C. A representative water penetration scan at 40 °C for this copolymer is shown in Figure 2.

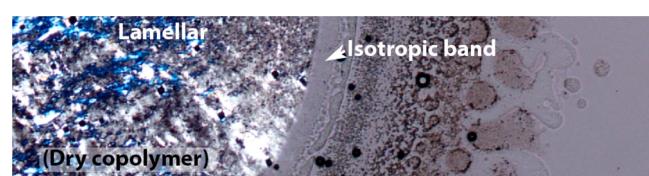


Figure 2. Water penetration scan of PEG10K₁₀PHYT₄₀ in excess water at 40 °C, displaying multiple bands (i.e. isotropic band). Dry copolymer is to the left of the figure, while the excess water region is on the right of the image. 100 \times magnification.

The self-assembly behavior of the copolymers was also assessed using SAXS, for two different systems, (i) with high polymer (>60% polymer content) and (ii) with high water (>60% hydration), to attempt to determine whether long-range order existed in the samples as an indication of formation of lyotropic liquid crystalline structure formation. No long-range order, typical of lyotropic crystalline phases, was detected during SAXS analysis of these systems. This indicated that the anisotropic and isotropic phases observed during CPLM were not able to persist at high hydration levels, and therefore are most likely type I (normal phases) phases. This is comparable to the behavior of F127, which has been reported to also form

Table 2. Visual Assessment of the Stability of Phytantriol Dispersions Using 1 wt % PEG-PHYT Copolymers As Steric Stabilizer^a

PEG MW (x)	PEG(x)-PHYT [mol %]				
	PEG(x) ₁₀ PHTY ₄₀	PEG(x) ₂₀ PHTY ₃₀	PEG(x) ₂₅ PHTY ₂₅	PEG(x) ₃₀ PHTY ₂₀	PEG(x) ₄₀ PHTY ₁₀
PEG200	—	—	—	—	—
PEG400	—	—	—	—	—
PEG600	—	—	—	—	—
PEG800	—	—	—	—/+	—/+
PEG1K	+	+++	+++	++	+++
PEG2K	++	+++	++	++	+++
PEG3K	+++	+++	+++	+	+++
PEG4K	++	+++	+++	+++	+++
PEG6K	++	++	++	+++	+++
PEG8K	+++	++	++	+	+
PEG10K	+++	++	++	+	+
PEG14K	++	++	+++	+	+

^aKey: +++, milky sample with no visible aggregates; ++, milky sample with few visible aggregates; +, milky/cloudy sample with aggregates; —, translucent sample with large aggregates [samples rated (—) were not progressed to ASA or SAXS assessment].

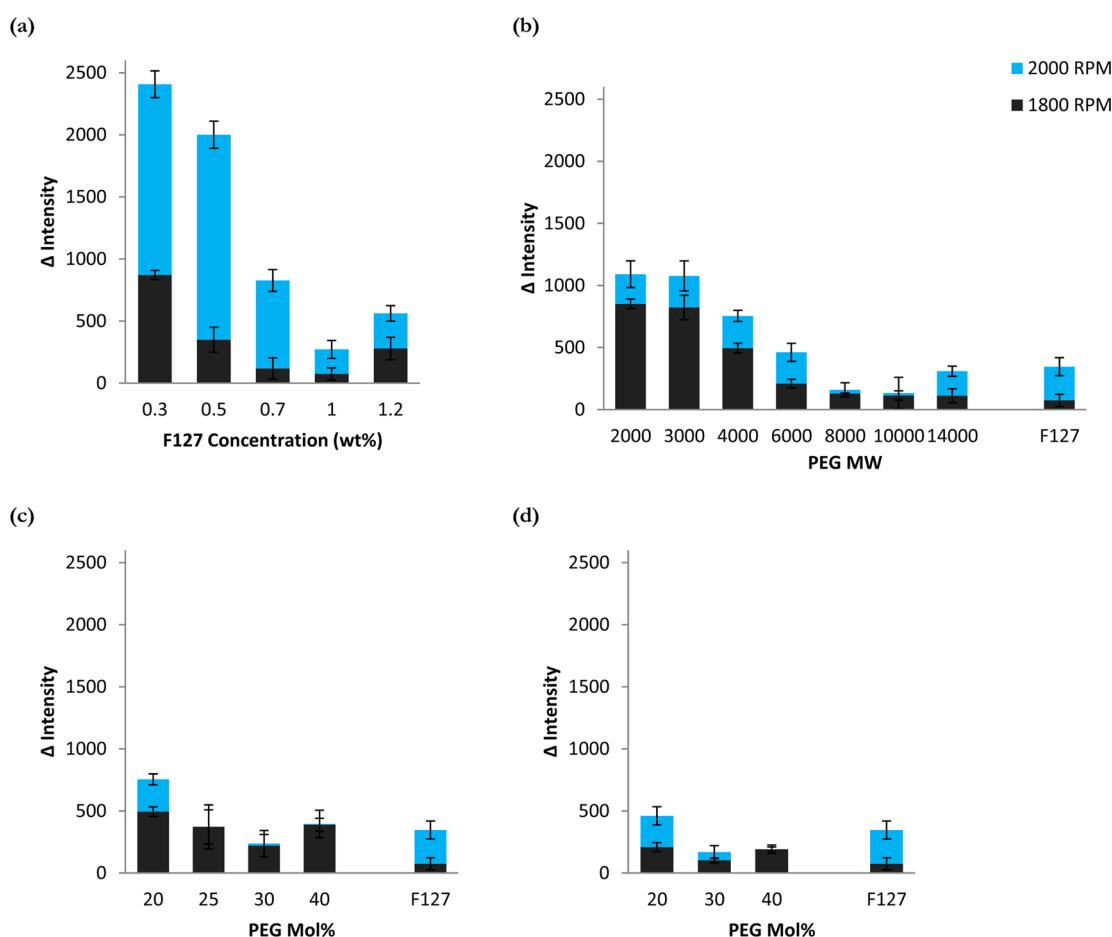


Figure 3. Accelerated stability assay results for (a) F127 (Control stabilizer) at 0.3, 0.5, 0.7, 1, and 1.2 wt % stabilizer concentration, (b) PEG₂₀PHTY₃₀ copolymer series, where PEG MW = 2K to 14K, (c) PEG4K-PHYT copolymer (from 20, 25, 30, and 40 PEG mol % copolymer series), and (d) PEG6K-PHYT copolymer (from 20, 30, and 40 PEG mol % copolymer series). ASA results after first spin at 1800 rpm are represented in gray columns, while ASA results after second spin at 2000 rpm are represented in blue columns. Steric stabilizer concentration for ASA results presented in (b)–(d) are 1 wt %, with control standard steric stabilizer F127 at 1 wt % presented to the right.

type I lyotropic liquid crystalline structures (isotropic phase and micellar cubic phase, Q_I).⁴²

Part II: Performance as Steric Stabilizers for Cubosomes. 3.3. Colloidal Stability. 3.3.1. Visual Assessment and DLS. A well-dispersed cubosome sample has a milky white,

aggregate-free appearance. This provides a good indication of stabilizer effectiveness. Dispersions which are translucent with visible aggregates are poorly stabilized. To perform a rapid initial screen of the stabilization capability of the copolymers, the phytantriol dispersions were assessed visually and

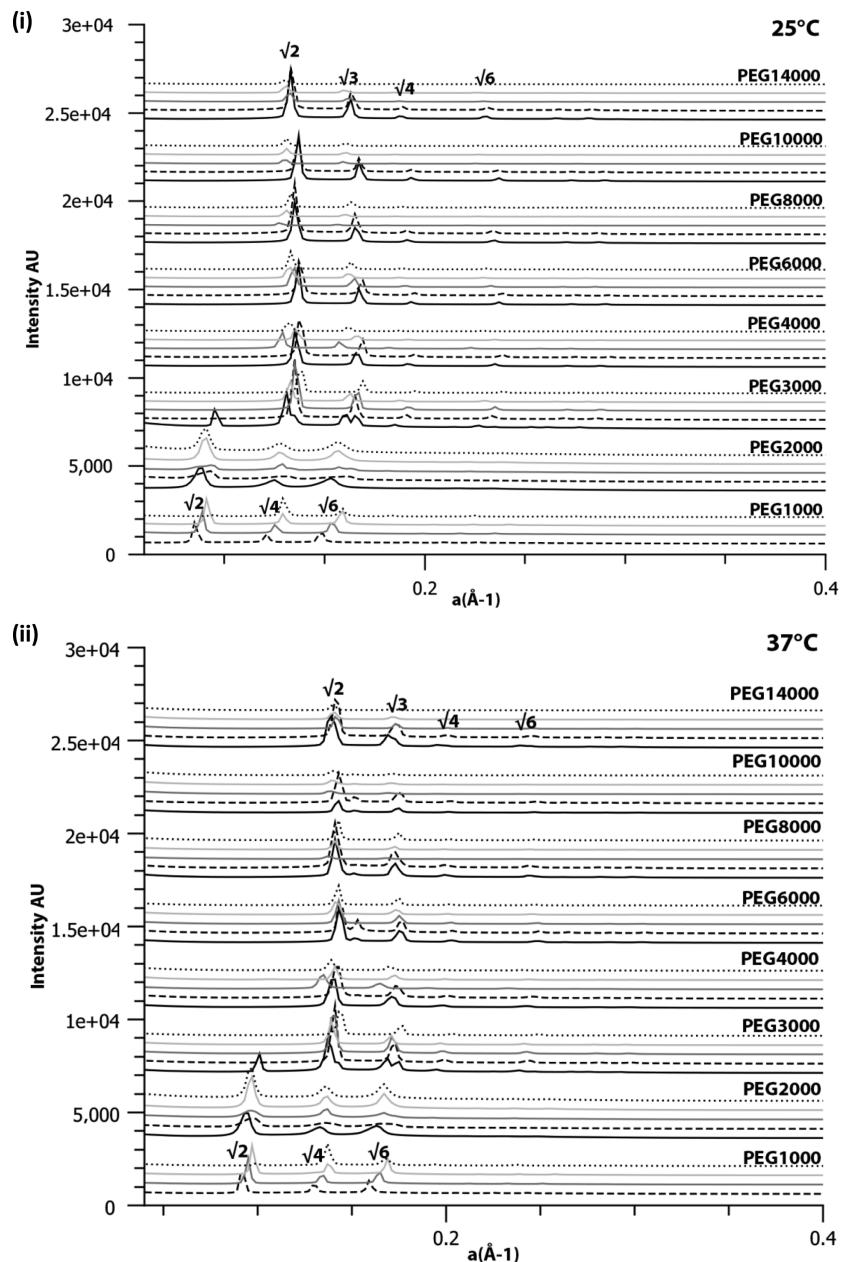


Figure 4. SAXS diffraction patterns at (i) room temperature ($25\text{ }^{\circ}\text{C}$) and (ii) physiological temperature ($37\text{ }^{\circ}\text{C}$) of phytantriol dispersions stabilized with PEG-PHYT copolymers where PEG mol %: 10% (1st, black line), 20% (2nd, black dash-line), 25% (3rd, dark gray line), 30% (4th, light gray line), 40% (5th, light gray dotted line), and PEG MW between 1K and 14K.

complemented with particle sizing measurements using dynamic light scattering. Visual assessment was performed on all dispersions, which were formed using 100 mg/mL phytantriol in PBS buffer solution, using 1 wt % of the new copolymers (Table 2).

It was found that copolymers which had a PEG MW $< 1\text{K}$ were unable to form stable dispersions, with large aggregates visible in the aqueous medium. Despite a slight cloudiness in the dispersions formed using $\text{PEG800}_{30}\text{PHYT}_{20}$ and $\text{PEG800}_{40}\text{PHYT}_{10}$, they were still poor quality dispersions with large visible aggregates (stability score $-/+$). It is apparent that the hydrophilic PEG chain $\geq \text{PEG1K}$ is required to create a steric barrier on the surface of the lyotropic liquid crystalline nanostructured particles for steric repulsion of neighboring colloids. In agreement with Kim et al., it was found that longer

hydrophilic chain lengths (i.e., $>\text{PEG1K}$) provided better stabilization.⁴³ Copolymers that were not able to stabilize particles were therefore excluded from further investigations. The remaining dispersions were found to have a similar “milky” appearance to those dispersions stabilized using F127 (the positive control known to effectively stabilize cubosomes).

The stability of the dispersions prepared using PEG6K copolymers was found to be dependent on the PEG molar ratio, with 10, 20, and 25 mol % scoring ++ and 30 and 40 mol % scoring +++. This indicates that a higher PEG to lipid ratio reduced the degree of aggregation. In contrast, copolymers with higher PEG molecular weights, such as PEG8K and PEG10K, displayed the reverse trend. Both PEG3K and PEG4K copolymers with 20, 25, and 40 mol % produced the most stable (+++) dispersions. Overall, the most stable dispersions

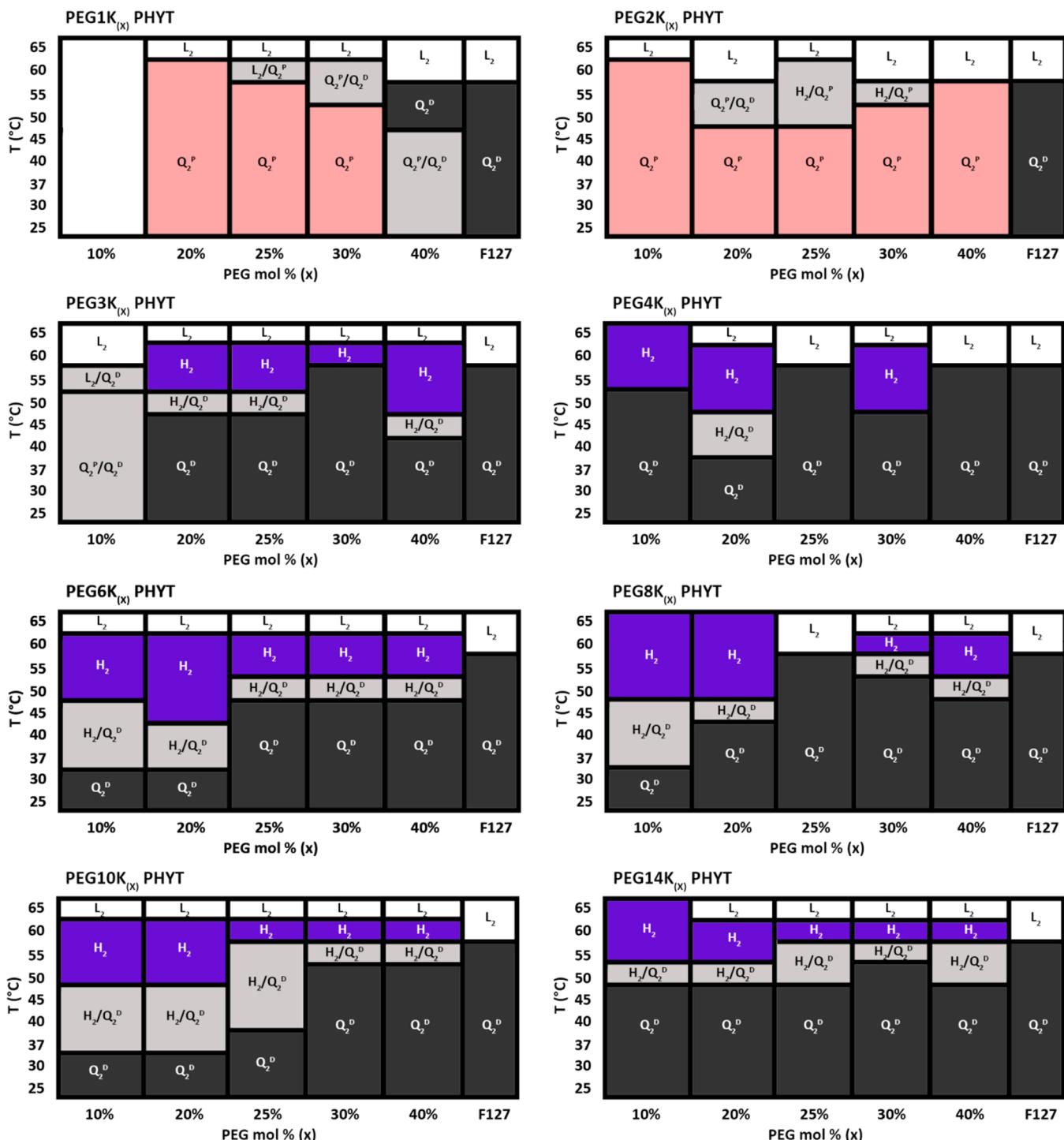


Figure 5. Lyotropic liquid crystalline (LLC) phases obtained during SAXS; temperature ($^{\circ}\text{C}$) vs PEG mol % over eight different PEG MWs within the five different copolymer series. Order of tables placed from low PEG MW (PEG1K) to high PEG MW (PEG14K). These tables show the effect of increasing PEG density (or respectively the decreasing lipid ratio in copolymer) on LLC phase behavior under increasing temperature (25–65 $^{\circ}\text{C}$).

were seen for the 20, 25, and 40 PEG mol % copolymer series, typically with PEG MW values between PEG1K and PEG6K.

Particle size and polydispersity index of the dispersions were measured using dynamic light scattering (see the Supporting Information for data). Although there are variations in the PEG content (i.e. varying PEG mol % or PEG MW) of the PEG-PHYT copolymer series employed to stabilize phytantriol dispersions, there was no discernible trend revealed by the

particle size measurements obtained that may highlight this. The average particle size of the dispersions measured using DLS was between 174 and 386 nm. It should be noted that DLS determination of particle size is not a clear indication of colloidal stability when measured in isolation because large phase-separating aggregates may not be detected by the technique, leading to a misleading average particle size distribution.

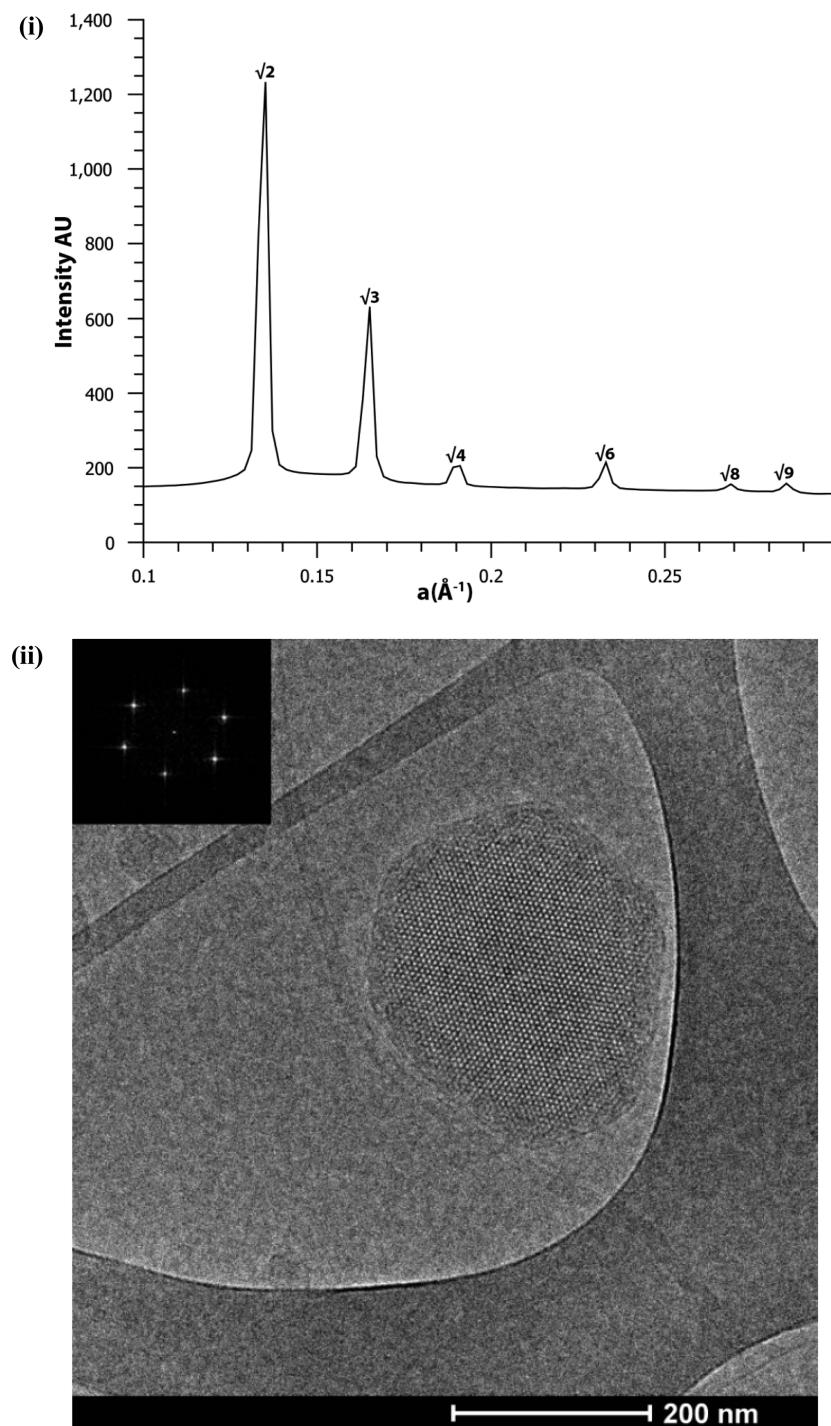


Figure 6. (i) SAXS diffraction pattern and (ii) cryo-TEM image observed in the [111] axis plane (inset shows FFT) of a phytantriol dispersion sterically stabilized using PEG6K₂₅PHYT₂₅ at 1 wt % stabilizer concentration.

3.3.2. Accelerated Stability Assay (ASA) for Steric Stabilizer Effectiveness. The effectiveness of PEG-PHYT copolymer as a steric stabilizer for stabilizing phytantriol dispersions was quantified with an accelerated stability assay, developed for the quantification of lyotropic liquid crystalline nanoparticle steric stabilizers compared to control stabilizer F127.⁴⁰ Steric stabilization of phytantriol cubosomes by F127 was assessed at 0.3, 0.5, 0.7, 1, and 1.2 wt % to provide comparison of systems to “poorly” (i.e., 0.3 wt %) and “well” (i.e., 1 wt %) stabilized systems based on previous experience with F127. Only

stabilizers with a visual assessment score of either ++ or +++ were assessed.

ASA results for F127 were in agreement with previous ASA results for this system,⁴⁰ with greater aggregation (i.e. increased creaming) correlating with increased fluorescence signal intensities. As expected, at 0.3 wt % F127, the greatest aggregation was observed during the ASA, correlating with the greatest increase in fluorescence signal. By increasing the F127 concentration, better steric stabilizer effectiveness was observed, with the optimal stabilizer concentration established to be 1 wt % (Figure 3).

With all the phytantriol dispersions stabilized at the same copolymer concentration of 1 wt %, a clear trend of decreasing change in fluorescence signal intensity was seen within the PEG₂₀PHYT₃₀ series with increasing PEG MW from 2K to 14K (Figure 3), with smaller changes in fluorescence signal intensity indicating better steric stabilizer effectiveness. Longer hydrophilic moieties (i.e. longer PEG chains) for the copolymer steric stabilizer provided better steric stabilization. This is illustrated in the PEG₂₀PHYT₃₀ copolymer series (Figure 3).

The accelerated stability assay results indicate that amphiphilic copolymers PEG-PHYT with larger PEG molar ratios, such as the copolymer series with 30 PEG mol %, are more effective steric stabilizers as a smaller change in fluorescence signal intensity was obtained. This is illustrated in Figure 3, where both PEG4K₃₀PHYT₂₀ and PEG6K₃₀PHYT₂₀ copolymers have the least change in fluorescence signal intensities.

ASA results also revealed that some of the copolymers provided comparable stability to that of F127 at 1 wt % (Figure 3). Specifically PEG6K₃₀PHYT₂₀ and copolymers from the PEG₂₀PHYT₃₀ stabilizers series, with PEG MW 8K to 14K.

3.3.3. Lyotropic Phase Behavior of Dispersions Stabilized with PEG-PHYT Copolymers. The PEG-PHYT copolymers were successful at sterically stabilizing inverse bicontinuous cubic phase nanostructured particles, at 1 wt % stabilizer concentration. All phytantriol dispersions stabilized at this concentration using PEG-PHYT copolymers, with PEG MW \geq 1K at 25 °C yielded a SAXS diffraction pattern indicative of a cubic (Q_2^D or Q_2^P) lyotropic liquid crystal phase (Figure 4). This corresponded to a Pn3m or Im3m space group symmetry, respectively. Cubosomes with a Pn3m space group symmetry, double diamond phase (Q_2^D), which is characteristic of the bulk phase formed by phytantriol in excess water, were stabilized by PEG-PHYT copolymers with PEG MW \geq 3K (i.e., \geq 68 PEG units on average) (Figure 4). These Q_2^D cubosomes had an average lattice parameter of 66.8 Å at 25 °C that decreased in size with increasing temperature. This is comparable to the lattice parameter recorded for phytantriol Q_2^D cubosomes stabilized by F127 (66.4 Å) at 25 °C. All the copolymers in the 25, 30, and 40 PEG mol % series, with PEG MW \geq 3K (i.e., \geq 68 PEG units on average) were able to stabilize cubosomes with a Q_2^D cubic phase at physiological temperature of 37 °C (Figure 4). Cubosomes with a primitive phase internal structure, Im3m space group symmetry (Q_2^P), were observed in phytantriol dispersions stabilized using PEG-PHYT copolymers with PEG MW \leq 2K (i.e., \leq 45 PEG units on average). These Q_2^P cubosomes had an average lattice parameter of 98.1 Å at 25 °C, which decreased with increasing temperature.

Phase transitions, such as from the cubic (Q_2^D) to the hexagonal (H_2) phase, can be induced by increasing the temperature of the lyotropic liquid crystal dispersion. The higher the phase transition temperature the more resilient the structure is to thermal changes. Higher phase transition temperatures were observed for phytantriol dispersions stabilized using PEG-PHYT copolymers, where the PEG block was of equal or greater molar ratio to the hydrophobic block (i.e. 25, 30, or 40 PEG mol % copolymer series). For example, phytantriol dispersions stabilized with the PEG₂₅PHYT₂₅ copolymer series, where PEG MW 3K-8K (i.e., 68-181 PEG units on average), displayed cubic to hexagonal phase transition temperatures greater or equal to 50 °C. The hexagonal (H_2) phase obtained in SAXS for the dispersions stabilized with the copolymers had an average

lattice parameter of 40.0 Å, which decreased with increasing temperatures. Copolymers in the 25 PEG mol % copolymer series exceeding PEG MW 8K (i.e., >181 PEG units on average) had relatively lower cubic to hexagonal transition temperatures, which were as low as 40 °C. This indicates for the 25 PEG mol % copolymer series, very high PEG lengths are not beneficial to maintain the particle cubic phase at moderate temperatures (i.e., \geq 40 °C).

The overall trend observed for the novel PEG-PHYT copolymer steric stabilizers, with PEG MW $>$ 2K (i.e., $>$ 45 PEG units on average), was that as the hydrophilic portion of the steric stabilizer structure was increased (i.e., $>$ 10 PEG mol % series) the higher the observed cubic to hexagonal phase transition temperature for phytantriol stabilized dispersions (Figure 5). The highest cubic to hexagonal phase transition temperatures (i.e. 55 °C) were observed for phytantriol dispersions stabilized with copolymers with PEG MW \geq 8K (i.e., $>$ 181 PEG units on average) from the PEG₃₀PHYT₂₀ and PEG₄₀PHYT₁₀ series. Thus, it was generally observed that the copolymer series with PEG molar ratios of 30 and 40 mol %, were able to maintain a stable Q_2^D cubic phase over a greater temperature range than the rest of the copolymers. In particular, the PEG₃₀PHYT₂₀ series, where PEG MW \geq 6K (i.e., \geq 136 PEG units on average), display both high cubic to hexagonal phase transition temperatures and ASA results that indicate it is an effective steric stabilizer, which is better than or comparable to the stabilizer effectiveness of standard control steric stabilizer F127.

Cryo-TEM was performed to further confirm the type of lyotropic liquid crystalline phase present in the aqueous dispersion of phytantriol. Cryo-TEM images taken of phytantriol nanoparticles stabilized with 1 wt % of PEG6K₂₅PHYT₂₅ in PBS at room temperature (25 °C) are shown in Figure 6. Cubosomes as well as vesicular structures were observed under cryo-TEM. Although cubosomes of different sizes were present, the majority of the nanostructured particles were approximately 200 nm in diameter. The Fourier transform of the internal structure of the particle (Figure 6 inset) shows a hexagonal arrangement. The internal structure is observed along the [111] axis, and the crystallographic planes observed are of the (110) type. This is compatible with the space group symmetries of Pn3m, Im3m, Ia3d, or H_{II}. However, considering the SAXS results of this sample, it is most likely the cubic structure with Pn3m space group symmetry. It should be noted that results from cryo-TEM analysis are not a complete/comprehensive representation of the entire sample size, as only a small fraction of the actual sample is examined under the microscope, and therefore results obtained are used to compliment the SAXS results/data, to determine lyotropic liquid crystalline behavior.

4. DISCUSSION

4.1. PEG-PHYT Copolymer Self Assembly Properties. Amphiphilic copolymers typically self-assemble in the presence of water to minimize interfacial free energy.^{44–46} The PEGylated-lipid (PEG-PHYT) copolymers self-assembled, with aggregation detected at 25 °C. These critical aggregation concentrations for the PEG-PHYT copolymers displayed a general trend, where the greater the proportion of lipophilic moiety (i.e. PHYT mol %) in the copolymer structure for a given PEG content, generally the lower the CAC value.

These findings were in reasonable agreement with the literature for micelle formation using AB diblock copolymers,

where the increase in the length of a hydrophobic block at a given length of a hydrophilic block causes a noticeable decrease in CMC value and increase in micelle stability.^{47–50} Although the copolymers in this study cannot be considered to be a diblock structure, their behavior is at least somewhat consistent with that of diblocks. The same trend applied to the copolymers with lower PEG molecular weights (i.e., <6K) within the same PEG molar ratio copolymer series. This trend is evident from the CAC values for the copolymer series with a PEG molar ratio > 10 mol %. These findings again were in general agreement with literature on micelle formation using AB diblock copolymers, where the increase in the length of a hydrophilic block at a given length of a hydrophobic block results in a small rise of the CMC value.^{47,49,51}

The general trend obtained in this study of higher CAC values for PEGylated-phytanyl copolymers with longer PEG lengths, is also in accordance to Rosen et al. where a series of 2-dodecyloxy poly(ethoxyethanol) surfactants were synthesized with different amounts of PEG (i.e., 2, 3, 4, 5, 7, and 8 PEG units) and higher CMC values were reported for surfactants with longer PEG chains.⁵² Rouxhet et al. also reported a similar trend in which higher CAC values were determined for the PEGylated-monoglyceride copolymers with longer PEG lengths (i.e., 9–48 PEG units on average). CMCs have been found to decrease strongly with increasing alkyl chain length of the surfactant.⁵³ Thus, in agreement with Kwon and co-workers for AB diblock copolymers, both the hydrophilic and hydrophobic blocks influence the micelle CMC value, with the hydrophobic block playing a more crucial role.^{48,54}

The Gibbs free energy of aggregation for the PEGylated-phytanyl copolymer series in this study was found to display a similar trend to the Gibbs free energy of micellation of the 2-dodecyloxy poly(ethoxyethanol) surfactant series reported by Rosen et al.,⁵² whereby greater negative ΔG_{agg} values were generally observed for surfactants with a lower PEG content and/or shorter PEG length (Figure 1). This general trend was most obvious in the PEG(x)₄₀PHYT₁₀ series, illustrated by Figure 1. Figure 1 displays an asymptotic relationship occurring between the copolymer's PEG length (i.e., number of PEG units) and its Gibbs free energy of aggregation, whereby increasing the copolymer's PEG length increases the value of Gibbs free energy of aggregation. However, increasing the copolymer's PEG length past 90 PEG units was found to incur little to no change to the value of the Gibbs free energy of aggregation, with a maximum ΔG_{agg} value of $-28.1 \text{ kJ mol}^{-1}$ reported.

The CAC values of known commercially-available cubosome steric stabilizers, Myrj 59 and F127, were also found to be similar to several of the novel PEGylated-lipid (PEG-PHYT) copolymers. In particular, the CAC for PEG6K₃₀PHYT₂₀ copolymer was $1.9 \mu\text{M}$, which is highly comparable to the CAC values of the commercial stabilizers, F127 ($2.1 \mu\text{M}$) and Myrj 59 ($1.8 \mu\text{M}$).⁸

The lyotropic liquid crystalline phase behavior displayed under CPLM by the PEGylated-lipid copolymers are consistent with the lyotropic liquid crystalline phase behavior reported by Fong et al. for monodispersed nonionic phytanyl ethylene oxide surfactants (Phytanyl(EO)_n, where $n = 1–8$) and nonionic isoprenoid-type hexahydrofarnesyl ethylene oxide surfactants (HFarnesyl(EO)_n, where $n = 1–8$), where hexagonal, lamellar (L_a) phase, inverse cubic (Q_2), and isotropic phases (L_2) were reported.^{55,56}

4.2. Steric Stabilization of Lyotropic Liquid Crystalline Nanostructured Particles.

Phytantriol-based cubosomes were successfully sterically-stabilized by the PEG-PHYT copolymers. SAXS results where PEG MW > 2K, at 1 wt % copolymer, the Q_2^D internal cubic phase was maintained at both 25 °C and physiological temperature, 37 °C, in agreement with the behavior of F127. The average particle size for the phytantriol Q_2^D cubosomes stabilized using the PEG-PHYT copolymers was between 200 and 300 nm in diameter, which is also comparable to phytantriol cubosomes stabilized with F127. Furthermore, the stability assessment by ASA showed that some of the copolymers (e.g., PEG30PHYT20 series, where PEG MW > 6K) provided steric stabilization for phytantriol cubosomes comparable to F127 at 1 wt % stabilizer concentration. This confirms that an essentially random copolymer structure can be as effective as an ABA triblock copolymer to provide steric stabilization for lyotropic liquid crystalline nanostructured particles. It is felt that the PEG-PHYT copolymers behave as block systems rather than random copolymers due to their amphiphilic nature. This type of behavior, displayed by random polymers, has also been reported before for random polymers with varying hydrophilic and lipophilic characteristics.⁵⁷

One of the important macromolecular features for a copolymer structure to be an effective steric stabilizer for cubosomes is having an asymmetrical amphiphilic structure containing a larger hydrophilic domain than the hydrophobic moiety (i.e., PEG₃₀PHYT₂₀ series, PEG MW $\geq 6\text{K}$). It was shown that copolymers that had a PEG MW < 1K were unable to successfully form dispersions, indicating the importance of PEG length when designing a steric stabilizer for cubosomes. For these PEGylated-lipid copolymers, a minimum of 22 ethylene glycol monomer units on average (i.e., PEG1K) was required for cubosomes to be stabilized (i.e., either Q_2^P or Q_2^D cubic phase). More specifically for obtaining phytantriol cubosomes with Q_2^D cubic phase, the copolymers were required to have at least 68 units on average of PEG (i.e., \geq PEG3K). These findings are comparable to results obtained using vitamin E TPGS, a known PEG-lipid stabilizer for phytantriol cubosomes.⁵⁸ Vitamin E TPGS, which has a PEG length of 22 PEG units on average (i.e. PEG1K), forms phytantriol dispersions with Q_2^P cubic internal structure, which is in accordance with the results herein where phytantriol dispersions stabilized using PHYT-PEG copolymers, with 22 PEG units on average, also formed Q_2^P cubosomes.

Pluronic stabilizers required a minimum of 26 PEG units on average to stabilize phytantriol dispersions with a cubic phase (i.e., either Q_2^P or Q_2^D cubic phase).¹⁶ To obtain the Q_2^D cubic phases, Pluronic stabilizers require at least 61 units on average of PEG in each hydrophilic arm, with a HLB value > 24 .¹⁶ Thus, regardless of whether the stabilizer is a PEGylated-lipid copolymer (i.e. PEG-PHYT) or triblock (i.e. PEG-PPO-PEG, Pluronic) copolymer, the minimum PEG length required to sterically stabilize phytantriol Q_2^D cubosome dispersions is ≥ 61 PEG units on average, with a HLB value > 17 . Similarly, when using Myrj (PEG-stearate) series to stabilize phytantriol cubosomes, the stabilizers with PEG length < 68 PEG units on average gave particles with Q_2^P internal structure, while ≥ 68 PEG units on average gave phytantriol cubosomes with Q_2^D structure.⁴⁰ The increased entropic effect of the 68 units on average (i.e., PEG3K) compared to 45 units on average (i.e., PEG2K) appears to be sufficient to stabilize phytantriol

nanostructured particles, even when using copolymers with a low PEG molar content.

A key factor to take into account for shorter PEG chain length PEG-PHYT copolymers causing a change in structure for phytantriol cubosomes, from the parent Q_2^D phase to the Q_2^P structure, is the localization of the polymer within the lipid matrix. PEG-PHYT stabilizers with 45 or fewer PEG units on average changes the mesophase of the dispersed nanostructured particles, indicating increased internalization of the stabilizer within the lipid matrix due to the shortened length of the PEG chain. These smaller amphiphiles may be able to penetrate into the particles more readily via the aqueous channels or by virtue of their greater hydrophobicity and hence partition tendency, driving the change in the mesophase. It is likely that this internalization results in less free amphiphile being available to provide surface coverage of the particles and therefore is consistent with the poorer colloidal stability. The poorer performance of the remainder of the shorter PEG-PHYT surfactants (i.e. ≤ 22 PEG units on average) may also be due to the decreasing PEG chain length, which would be expected to have a reduced capacity to create sufficient steric hindrance to inhibit flocculation.

Phytantriol dispersions stabilized with copolymers consisting of a longer hydrophilic moiety, $PEG_{30}PHYT_{20}$ series, where PEG MW $\geq 6K$, resulted in higher cubic to hexagonal phase transition temperatures (i.e., >50 °C). Furthermore, these copolymers were effective steric stabilizers for phytantriol dispersions, illustrated and quantified using the ASA screening experiments (Figure 3), without compromising structure formation.

As well as establishing that the novel steric stabilizer copolymer (PEG-PHYT) requires a minimum PEG length to establish steric stability of lyotropic liquid crystalline nanostructured particles, it should also be noted that it can be disadvantageous to make a steric stabilizer “too” hydrophilic (i.e., 40 PEG mol % copolymer series). For example, the dispersions which were stabilized with copolymers in the $PEG_{40}PHYT_{10}$ series, where PEG MW is 4K and 6K, displayed ASA results with lower steric stabilizer effectiveness than those stabilized with a lower PEG molar ratio (i.e., $PEG_{30}PHYT_{20}$ series) at the same PEG length (Figure 3). In addition to having a lower steric stabilizer effectiveness, phytantriol dispersions stabilized with copolymers from the $PEG_{40}PHYT_{10}$ series were also shown to have lower cubic to hexagonal phase transition temperatures than those stabilized using stabilizers from the $PEG_{30}PHYT_{20}$ series (Figure 5). This indicates that the copolymer stabilizer series does appear to have a maximum “hydrophilic threshold” (i.e., >30 PEG mol %) at which the effectiveness of the steric stabilizer is compromised when exceeded. The diminished steric stabilizer effectiveness by copolymers with an extremely large hydrophilic domain (i.e., $PEG_{40}PHYT_{10}$ series) could be due to (i) the water solubility being too high and the stabilizer not adsorbing sufficiently to the particle surface or (ii) flocculation of the particles caused by “bridging” mechanisms by the extended PEG chains of neighboring particles interacting with each other.⁵⁹ Thus, it is recommended that when designing a steric stabilizer copolymer structure, the hydrophilic moiety should not exceed 60% of the total amphiphilic structure. The optimal PEG-PHYT copolymer stabilizer parameters (i.e., PEG length and mol %) lie between 68 and 136 PEG units on average and between 20 and 40 PEG mol %, for producing good quality dispersions (i.e.,

devoid of aggregates), which maintain a Q_2^D cubic phase at 37 °C (see the Supporting Information).

Having established the design rules for copolymer-based stabilizers that provide good quality stable dispersions while retaining the parent internal phase structure, future efforts will be directed toward preparing copolymers with more defined structures using controlled polymerization approaches such as reversible addition–fragmentation chain transfer (RAFT)^{37,60} and the inclusion of functionalizable monomer units to facilitate attachment of targeting ligands to drive the progress in the cubosome field from “static” delivery particles, to biologically interactive delivery systems better suited for theranostic applications.

5. CONCLUSION

The novel PEGylated-phytanyl copolymers displayed self-assembly properties in water. A select number successfully sterically stabilized cubic lyotropic liquid crystalline nanostructured particles. Application of these amphiphilic PEGylated-lipid copolymers in other lipid-based self-assembly systems seems prospective. As lyotropic liquid crystalline nanoparticles (i.e. cubosomes) are further developed for biomedical applications, developing steric stabilizers with optimal performance is a crucial part. The amphiphilic copolymer PEG-PHYT series synthesized in this study has illustrated the potential of using customized steric stabilizers. Equivalent steric stabilizer effectiveness comparable to “gold standard” Pluronic F127 can be achieved using this novel copolymer series.

■ ASSOCIATED CONTENT

S Supporting Information

Data sets obtained of the NMR, CAC, CPLM, CAC, DLS, and SAXS results for PEG-PHYT copolymers and their phytantriol dispersions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

C.J.D. was the recipient of an Australian Research Council (ARC) Federation Fellowship. J.Y.T.C. was the recipient of an Australian Postgraduate Award and a CSIRO Ph.D. studentship. X.M. was the recipient of a CSIRO-Monash University Collaborative research postdoctoral fellowship. B.J.B. is the recipient of an Australian Research Council (ARC) Future Fellowship (FT120100697). This research was undertaken in part at the SAXS/WAXS beamline at the Australian Synchrotron, Victoria, Australia.

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