Accelerated Stability Assay (ASA) for Colloidal Systems

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S *Supporting Information*

ABSTRACT: Assessment of the stability of colloidal systems, in particular lyotropic liquid crystalline dispersions, such as cubosomes and hexosomes, is typically performed qualitatively or with limited throughput on specialized instruments. Here, an accelerated stability assay for colloidal particles has been developed in 384-well plates with standard laboratory equipment. These protocols enable quantitative assessments of colloidal stability. To demonstrate the applicability of the assay, several steric stabilizers for cubic phase nanostructured particles (cubosomes) have been compared to the current "gold standard" Pluronic F127.

KEYWORDS: *cubosome, lyotropic liquid crystalline nanoparticle, colloidal stability, steric stabilization, accelerated stability assay*
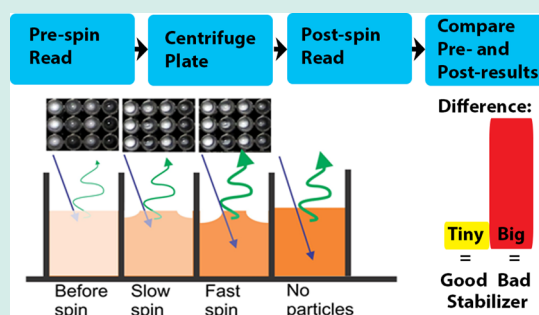
Lyotropic liquid crystalline nanostructured particles have been explored for their potential in drug delivery systems. Liposomes, based on a 1D lamellar lyotropic liquid crystalline structure, have been extensively used as drug delivery vehicles with 12 clinically approved liposomal drug formulations on the market and twenty-two liposomal drugs undergoing clinical trials.[1,2] Particles based on other lyotropic liquid crystalline structures, such as cubosomes (dispersed inverse bicontinuous cubic phase) and hexosomes (dispersed inverse hexagonal phases) are being developed as potential drug delivery systems. The key advantages to these nanostructured particles with complex internal structure include their potential for controllable release and their increased lipid volume fraction per particle, which provides a lipophilic area for containing poorly water-soluble therapeutics.[3,4] These dispersed drug delivery systems typically consist of the lipid matrix, and a steric stabilizer, which provides the dispersion with varying degrees of stability, depending on the lipid and stabilizer combination and concentrations.[5,6]

The steric stabilizer is a key component of nanostructured particle preparation, particularly in the case of cubosomes and hexosomes where the internal interfacial area is high and may sequester stabilizer otherwise available for colloidal stability.[7] These cubosomes and hexosomes often consist of phytantriol as the core lipid matrix (Figure 1). A quantitative measure is required to assess the effectiveness of the steric stabilization conferred to lyotropic liquid crystalline nanostructured particles. Typically, the stabilizers used are amphiphilic polymers with the hydrophilic region consisting of one or more poly(ethylene glycol) (PEG) domains (e.g., Tween and Cremophor stabilizers). PEG has been shown to be able to

form a stealth corona around liposomes, significantly reducing the rapid uptake of intravenously injected particulate drug carriers by cells of the mononuclear phagocyte system (MPS).[8,9] The most frequently used steric stabilizer for cubosomes and hexosomes is the nonionic triblock copolymer Pluronic F127. It consists of two blocks of PEG (100 monomer units on average) on either side of a poly(propylene oxide) domain (65 monomer units on average) (Figure 1). Pluronic F108 and Myrj 59 (PEG100-stearate), also represented in Figure 1, stabilize inverse bicontinuous cubic phases of phytantriol dispersions as, or more efficiently than Pluronic F127.[5,6]

Despite the advent of high-throughput preparation and characterization techniques to identify the effect of the steric stabilizer on lyotropic liquid crystal phase behavior,[3] there has so far been no assay available to screen new steric stabilizers over a large range of concentrations to quantifiably assess their effectiveness. The current norm is to use a visual assessment to determine if particle aggregation has occurred. This technique, however, is only suitable for differentiating poor stabilizers from stabilizers which are capable of producing milky dispersions void of aggregates. Dynamic light scattering may also be used to detect changes in particle size, however large aggregates (>1 μm) are typically difficult to detect as they fall outside of the detection range of typical laboratory instruments. Dynamic light scattering is therefore not a reliable measure of particle
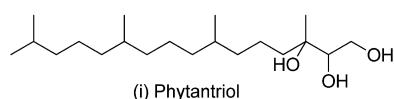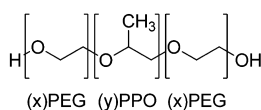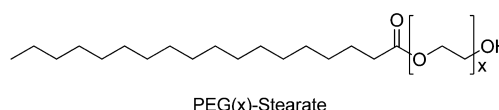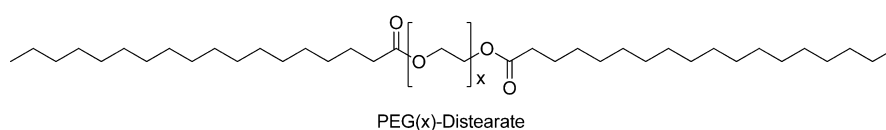
Figure 1. Chemical structures of (i) Phytantriol, (ii) Pluronic F127 (control steric stabilizer), (iii) Pluronic F108, (iv) PEG100-stearate, (v) PEG150-stearate, and (vi) PEG150-distearate.

stability as it may measure a net reduction in average size despite the presence of aggregates. At present, dispersion stability can be quantified by using a microprocessor-controlled analytical centrifuge that detects demixing phenomena (e.g., particle aggregation and clarification) of the dispersed systems during centrifugation over the whole sample length.[10,11] However, this is a low-throughput technique. Using a similar principle, detection of particle aggregation or clarification on centrifugation, herein we demonstrate the use of a fluorescence-based reproducible, alternative accelerated stability assessment technique which uses standard laboratory instruments (i.e., 96- or 384-well plate reader and plate centrifuge) to assess colloidal stability (Figure 2). A fluorescence approach was taken because of its high sensitivity to changes of sample stability, high signal to-noise and high reproducibility.

The principle of this assay is that the intensity of fluorescence measured is proportional to the quantity of particles dispersed in the solution. Centrifugation of the samples drives particle aggregation and these concentrate at the air water interface of the sample well. This is due to the low density of the lipid rich domain. Aggregation preferentially occurs on the edges of the multititer plate wells. The decrease in particle concentration in solution leads to reduced excitation and emission light scattering which results in an increase in emission signal in poorly stabilized samples. The magnitude of the change in fluorescence intensity following sample centrifugation correlates to particle aggregation and permits the quantification of the relative effectiveness of particle stabilization. Stable systems in which little aggregation occurs can be identified due to minimal change in fluorescence signal intensity before and after centrifugation (Figure 2).

The ability of this assay to quantifiably assess stabilizer performance is demonstrated by testing several steric stabilizers
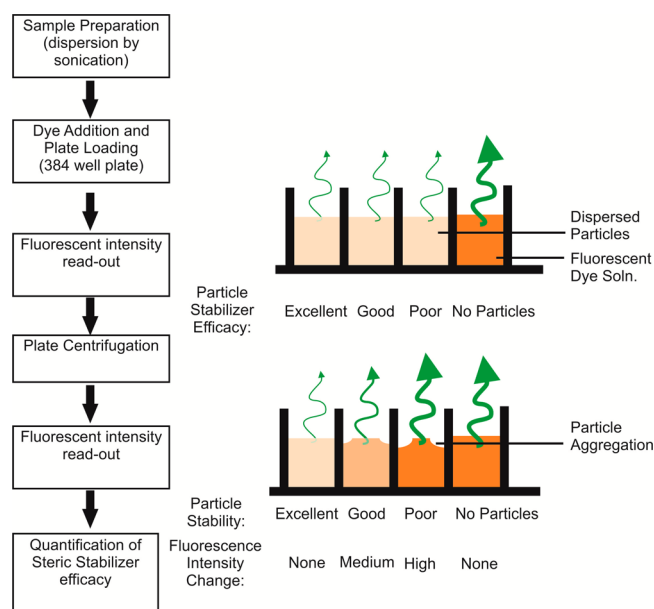

Figure 2. Schematic of the accelerated stability assay.

including Pluronic F108, Myrj 59 (PEG100-stearate), PEG150-stearate and PEG150-distearate, against Pluronic F127 as control (Figure 1). PEG150-stearate has a PEG domain of 150 monomer units on average compared to the 100 monomer units of Myrj 59 on average. In contrast to PEG150-stearate, PEG150-distearate is a molecule with two hydrophobic domains, one at each end of a PEG domain which is 150 monomer units long on average. Amphiphilic block config-uration (i.e., terminal block ends using hydrophobic or hydrophilic blocks) of triblock copolymers used for steric

stabilization may play an important part in stabilizer effectiveness.

The aim of this assay was to rapidly verify the effectiveness of a steric stabilizer for colloidal lyotropic liquid crystalline dispersions. Stabilizer effectiveness is dependent on a wide range of parameters, which includes stabilizer concentration, stabilizer structure (i.e., length of the amphiphilic domains, such as PEG length), temperature and buffer pH. This assay permits investigators to screen the effectiveness of steric stabilizers using centrifugation to accelerate the aggregation process within colloidal systems. Because of the relative densities of the aggregated lipid domain and the aqueous domain, this typically results in particle aggregation at the sample surface. As illustrated by Figure 2, particle aggregation occurred around the edge of the well, on the surface of the sample after centrifugation. Typically the longer centrifugation time and greater the spin speeds, the larger the dispersion destabilization observed.

Accelerated Stability Assay (ASA) with Different Concentrations of Pluronic F127. Demonstration of the discriminatory power of this assay was demonstrated using Pluronic F127, for inverse bicontinuous cubic phase dispersions (cubosomes) made up of phytantriol, because the dependence of stability on stabilizer concentration is well characterized. The ASA should differentiate quantifiably between known "poorly" stabilized (3 wt % Pluronic F127) and adequately stabilized (10 wt % Pluronic F127) dispersions. Consequently the colloidal stability of phytantriol cubosomes stabilized with Pluronic F127 at 3, 5, 7, 10, and 12 wt % stabilizer concentrations was tested using the ASA assay.

A change in fluorescence signal intensity from post and prespin cycles at 1800 rpm for 5 min was most evident for those with lower stabilizer concentrations (Figure 3). The magnitude of the fluore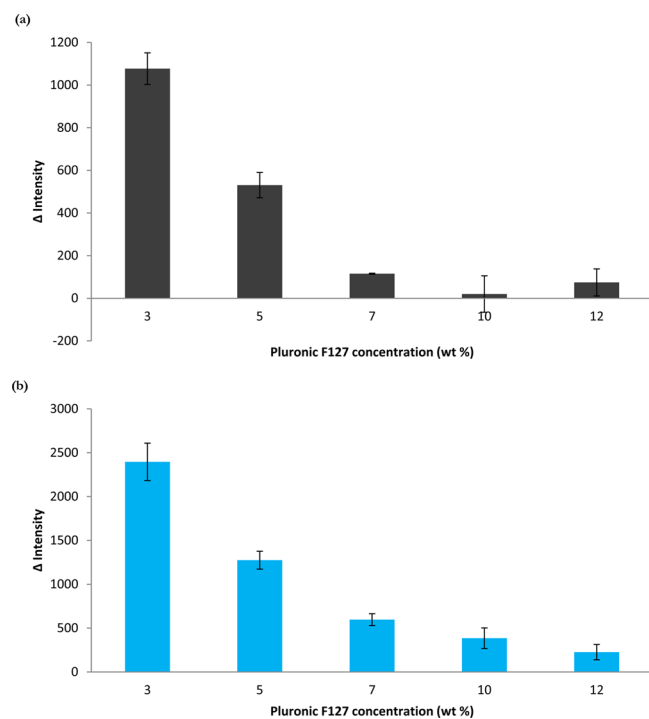scence signal intensity change decreased with increased stabilizer concentration and therefore enhanced stability. For example, the change in fluorescence signal intensity for dispersions stabilized with 3 wt % Pluronic F127 was 1000 A.U. For the more stable dispersions (10 and 12 wt % Pluronic F127/phytantriol dispersions), this change was reduced by at least an order of magnitude. The magnitude of the change in fluorescence signal intensity is inversely proportional to particle stability. These reproducible and discernible changes make this assay suitable for the assessment of particle stability.

The fact that otherwise stable dispersions can be destabilized through further centrifugation means that this assay should be applicable to a range of systems. A second centrifugation spin at 2000 rpm for 5 min gives rise to further colloidal instability. The change in signal intensity doubled for all dispersions, while retaining the same inversely proportional trend with regard to stabilizer concentration seen after the first spin at 1800 rpm (Figure 3). This assay, with experiments performed in triplicates, is therefore adaptable to the differential effectiveness of stabilizers simply by increasing centrifugal speed or time.

It is imperative that to assess the stability of other steric stabilizers, a standard control calibration (in this case Pluronic F127) be present as part of the ASA performed. This was particularly important as the fluorescence detection system is sensitive to parameters such as concentration, bleaching and environmental variables. The ASA developed here demonstrates a practical accelerated method using standard laboratory instruments that has the ability to compare large data sets of samples (i.e., multiwell plate) in order to determine the steric stabilization effectiveness of various steric stabilizers compared to Pluronic F127.

Real Time Intrinsic Colloidal Stability. Aggregation is inevitable in colloidal systems that are not thermodynamically stable. Phytantriol dispersions stabilized with Pluronic F127 has poor stability at very low stabilizer concentrations (i.e., 1 wt %) shortly after sonication. To ensure that the ASA reflects the likely stability that would be expected to occur over time without centrifugation, but at accelerated time scales, a comparable experiment was performed using identical conditions and reagents with only the centrifugation aspect excluded. For this set of dispersions (phytantriol cubosomes stabilized with Pluronic F127 at 3, 5, 7, 10, and 12 wt %), fluorescence was measured daily over a two week time period. At 24 h following preparation, the dispersions with the lower stabilizer concentration were showing higher changes in signal intensity compared to the higher 10 wt % stabilizer concentration typically used in cubosome preparation (Figure 4). Over time, all samples produced higher fluorescence signal intensities, representative of gradual increase in particle aggregation occurring within all the samples. The changes in signal intensity were comparable to those obtained with the
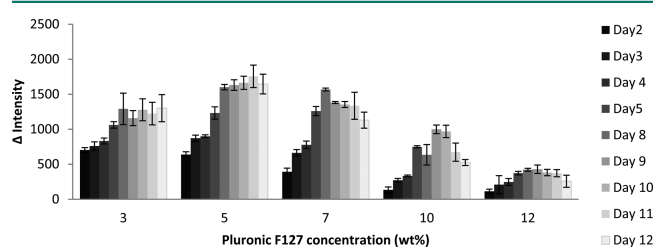

Figure 3. Pluronic F127 concentration vs Δ intensity after (a) 1800 rpm, (b) 2000 rpm for 5 min.

Figure 4. Pluronic F127 fluorescence signal intensity change progression with time (2 weeks).

accelerated stability assay demonstrating its validity. In addition to increasing fluorescence signal intensities, visual cues of particle aggregation occurring in phytantriol cubosome systems over time also occurred for all five stabilizer concentrations, 3, 5, 7, 10, and 12 wt %, contained in 1.5 mL microcentrifuge tubes (see Supporting Information).

Using ASA to Compare Alternative Steric Stabilizers with Pluronic F127. To demonstrate the potential of this assay, we also quantified the performance of a range of previously reported and new steric stabilizers[5,6] Phytantriol dispersions stabilized using Pluronic F108, Myrj 59 (PEG100-stearate), PEG150-stearate, and PEG150-distearate, were made with the stabilizer concentrations that contained the same amount of PEG moles used in Pluronic F127 at 3, 5, 7, 10, and 12 wt % stabilizer concentrations. These concentrations were doubled for PEG-stearate dispersions to account for the two PEG blocks in Pluronic copolymers. It was determined that at the same molar concentration of PEG to Pluronic F127, Pluronic F108 had reduced change in fluorescence signal intensity, thus indicating that it has better stabilizer effectiveness than Pluronic F127 (Figures 5 and 6).
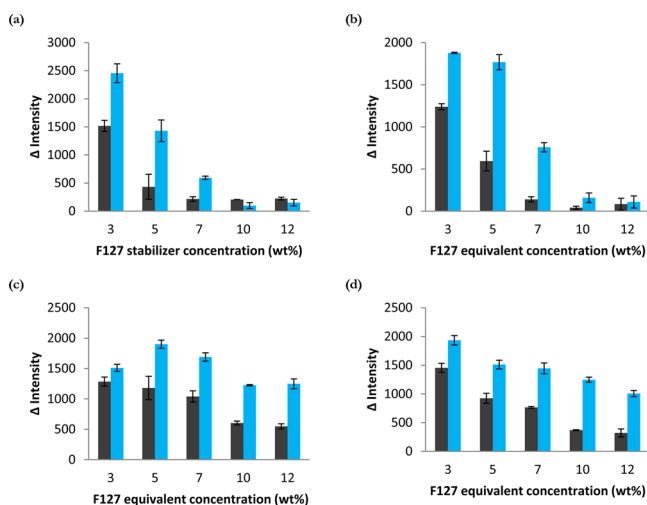
Figure 6. ASA graph of alternative steric stabilizers compared with Pluronic F127 after (a) first spin of 1800 rpm and (b) second spin of 2000 rpm. *X*-axis shows stabilizer concentration equivalent to PEG molar mass of control stabilizer, Pluronic F127 wt % concentrations.

Figure 5. ASA graphs of (a) Pluronic F127−control steric stabilizer, (b) Pluronic F108, (c) PEG100-stearate (Myrj 59) and (d) PEG150-stearate. *X*-axis shows stabilizer concentration equivalent to PEG molar mass of control stabilizer, Pluronic F127 wt % concentrations. Results after the first spin 1800 rpm represented by left column (gray color). Results after second spin 2000 rpm represented by right column (blue color).

By contrast, dispersions stabilized with PEG-stearates (i.e., Myrj) displayed greater changes in fluorescence signal intensity compared to Pluronic F127, suggesting Pluronic F127 has better steric stabilizer effectiveness than both PEG100-stearate and PEG150-stearate for phytantriol dispersions. However, when comparing steric stabilizer performance within the PEG-stearate series, it was found that increasing the hydrophilic domain, or PEG length, from 100 monomer units on average (i.e., PEG100-stearate) to 150 monomer units on average (i.e., PEG150-stearate) improved the performance of the steric stabilizer. This is shown in Figures 5 and 6, where the results of dispersions stabilized with PEG150-stearate displayed smaller changes in its fluorescence signal intensity than dispersions stabilized with PEG100-stearate. Similarly to the Pluronic series, increasing the PEG length (i.e., > 100 PEG units) within the PEG-stearate steric stabilizer series improved the steric
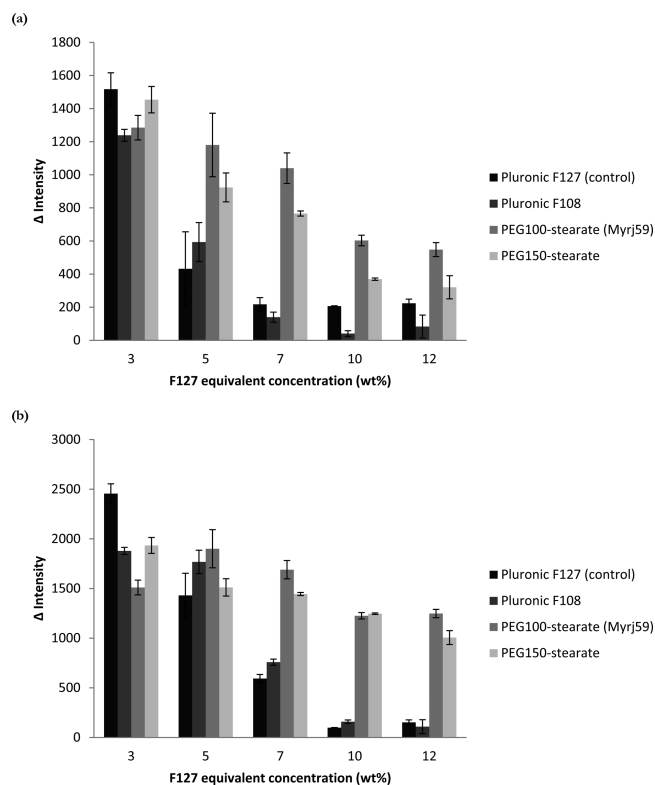
stabilizer's effectiveness for stabilizing phytantriol dispersions. Furthermore, SAXS and cryo-TEM results confirmed that PEG150-stearate stabilized inverse bicontinuous (Q_2^D) phytantriol cubosomes in excess water (see Supporting Information).

Steric stabilizer concentration was also found to influence the effectiveness of the alternative steric stabilizers. As expected increasing stabilizer concentration improves the steric stabilizer's performance (Figure 5 and Figure 6).

A practical, reproducible accelerated stability assay for assessing stability of colloidal systems was reported, using a fluorescence plate reader and plate centrifugation equipment. The use of multiwell plates (i.e., 384-well plates) allows for accelerated methodologies to be pursued. This particular methodology has been optimized for quantification of the steric stabilizer effectiveness in cubic lyotropic liquid crystalline nanostructured particles, the protocols may, however, be adapted for quantifying stability in other colloidal and nanostructured particulate systems.

■ EXPERIMENTAL PROCEDURES

Materials. Pluronic F127, fluorescein sodium salt, Myrj 59 and 0.01 M phosphate buffered saline (PBS) solution (pH 7.4) were purchased from Sigma-Aldrich, Sydney, NSW, Australia. Phytantriol (3,7,11,15-tetramethylhexadecane-1,2,3-triol) was a gift from DSM Nutritional Products, Sydney, NSW, Australia. PEG-150-stearate and PEG-150-distearate were generously provided by HallStar Co., Bedford Park, Illinois, U.S.A. Pluronic F108 was a kind donation from Prof. Joe McGuire, Oregon State University, U.S.A. All chemicals were used without further purification.

Preparation of Nanostructured Particles. Lyotropic liquid crystalline phase dispersions were prepared at a concentration of 100 mg/mL of phytantriol in 500 μL of 0.01 M PBS buffer solution, with 3, 5, 7, 10, and 12 wt % of steric stabilizer Pluronic F127. The range of Pluronic F127 stabilizer concentrations were selected to provide a variety of known poorly to excellently stabilized systems. Briefly, 50 mg of phytantriol was placed into each of five 1.5 mL centrifuge tubes. To each sample a Pluronic F127 steric stabilizer solution was added. The concentrations of Pluronic F127 tested were 3, 5, 7, 10, and 12 wt % (with respect to lipid) dissolved in 0.01 M PBS buffer solution. Samples were sonicated using a Misonix Ultrasonic Liquid Processor Microtip Probe Sonicator (Misonix Inc., NY, U.S.A.), with a 418 Misonix probe. The sequence programmed for the sonication of samples consisted of three programs, run in succession: 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. The sample temperature during sonication was monitored to prevent overheating of samples. The sample sonication temperature was observed to be consistent between 65 and 70 °C, during pulse sonication of samples.

Lyotropic liquid crystalline dispersions stabilized using the new stabilizers PEG150-stearate and PEG150-distearate were characterized using small-angle X-ray scattering (SAXS) and cryo-transmission electron microscopy (cryo-TEM). Briefly, the SAXS samples were loaded in special 1.5 mm capillaries (Hampton Research, U.S.A.) and positioned in a custom-designed capillary holder capable of holding 34 capillaries 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 at 25 and 37 °C. The exposure time for each sample was 1 s. SAXS data was analyzed using an IDL-based AXcess software package.[12]

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. 200-Mesh copper grids coated with perforated carbon film (Lacey carbon film, ProSciTech, Queensland, Australia) were glow discharged in nitrogen to render them hydrophilic. Four microliter aliquots of the sample 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, U.S.A.) 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 Megaview III CCD camera and AnalySIS camera control software (Olympus) using magnifications in the range from 30 000× to 97 000×.

Accelerated Stability Assay (ASA) Optimization. A batch of poorly stabilized phytantriol dispersions (3 wt % stabilizer concentration) and standard phytantriol dispersions (10 wt % stabilizer concentration) were made to establish significant differences between the two extremes of colloidal

stability. It should be noted that 1 wt % stabilizer was not used for the "poor stabilizing concentration" as the sample was required to be sufficiently dispersed and free of large aggregates. A 1:1 serial dilution of 10 mg/mL fluorescein dye in 0.01 M PBS solution was made with twenty-three sequential dilutions, resulting in the following dye concentrations: 10, 5, 2.5, 1.25, 6.3×10^{-1}, 3.1×10^{-1}, 1.6×10^{-1}, 7.8×10^{-2}, 3.9×10^{-2}, 2.0×10^{-2}, 9.8×10^{-3}, 4.9×10^{-3}, 2.4×10^{-3}, 1.2×10^{-3}, 6.1×10^{-4}, 3.1×10^{-4}, 1.5×10^{-4}, 7.6×10^{-5}, 3.8×10^{-5}, 1.9×10^{-5}, 9.5×10^{-6}, 4.8×10^{-6}, 2.4×10^{-6}, and 1.2×10^{-6} mg/mL. Fluorescein was used as a fluorescent dye due to its water solubility in PBS which results in minimal interaction with the lipid nanoparticles. Each dye solution was mixed at equal volumes with either a "poorly" stabilized (3 wt % stabilizer concentration) or the standard (10 wt % stabilizer concentration) phytantriol dispersion and pipetted into a Corning low volume, 384 round-well, black, with clear flat bottom, polystyrene microplate (product no. 3540). Adhesion of colloidal samples to the multititer plate walls will be sample dependent and should be taken into account during the application of this methodology. The optimization of this stability assay included testing plates with different well geometry (i.e., round or square). It is important to note that the round well geometry was found to be an important factor in the assay, as data obtained from plates with circular wells yielded highly reproducible data compared to square well geometries. Three repeats were made for each dye and cubosome mixture. Control samples consisted of three repeats of PBS and cubosome mixtures, as well as PBS and dye wells. Measurements of fluorescence intensity at emission wavelength of 530 nm, were taken pre- and postcentrifugation using a top-reading FlexStation 3 Multimode microplate reader (Molecular Devices Company, CA, U.S.A.), and processed on SoftMax Pro software. It is important to note that the top-read setting on this instrument is vital to obtaining usable ASA data sets, as the effect of particle aggregation after centrifugation occurs at the surface of the sample (top-end) of the well (Figure 2), and is only detected when using the "top-read" and not the "bottom-read" mode. The plate was spun at 645g (1800 rpm) for 5 min using Heraeus Multifuge ×3 Centrifuge (Thermo Scientific, Germany). The optimized dye concentration for the ASA was 3.1×10^{-4} mg/mL of fluorescein sodium salt in 0.01 M PBS buffer solution.

Final Accelerated Stability Assay (ASA) Protocol. Lyotropic liquid crystal phytantriol dispersions stabilized with 3, 5, 7, 10, and 12 wt % of Pluronic F127 were mixed at equal volumes with dye solution (i.e., 15 μL cubosome sample mixed with 15 μL dye solution) and pipetted into a 384 black round-well Corning plate. The same was done using PBS buffer solution instead of dye solution (3.1×10^{-4} mg/mL) for control samples. Negative control samples consisted of PBS buffer and dye solution. A minimum of three repeats were performed for each cubosome mixture with dye solution or PBS buffer solution. Fluorescence signal intensities were taken pre- and postcentrifugation (Figure 2). The centrifugation of plates was performed with a Heraeus Multifuge ×3 Centrifuge (Thermo Scientific, Germany). Fluorescence signal measurements were taken as described above. The plate was initially spun at 645g (1800 rpm) for 5 min, measured for fluorescence signal and then respun at 796g (2000 rpm) for 5 min. It was found that centrifugation speeds greater than 2000 rpm resulted in particle aggregation of all dispersions, resulting in less differentiation between "poor" and "well" dispersed

samples, and results from lower speeds (i.e., <1000 rpm) were inconsistent and did not always result in particle aggregation of poor samples, which also led to less differentiation between the poor and well dispersed samples.

Intrinsic Colloidal Stability Study (i.e., Real-Time Nonaccelerated Stability Study). The aim of this stability assay was to accelerate the destabilization of dispersed liquid crystalline dispersions that would naturally occur with time. To ensure that the results obtained herein correlate with the effects of particle destabilization over time, dispersions were left to age without centrifugation and sample fluorescence measured. Samples of phytantriol dispersions in 0.01 M PBS buffer solution, stabilized with 3, 5, 7, 10, and 12 wt % of steric stabilizer Pluronic F127 were assessed over a two week time duration for any changes (i.e., particle aggregation). Samples were mixed with an equal volume of dye solution and pipetted into a 384 black round-well Corning plate. Control samples were mixed with equal volumes of PBS buffer solution. Additional controls such as PBS buffer solution and pure dye solution were tested, to monitor the natural decrease of the fluorescence signal from the dye over time using the same fluorescence method described above.

ASSOCIATED CONTENT

Ⓢ Supporting Information
Data sets obtained for the ASA fluorescence measurements, visual record of the time deterioration of stability study, and SAXS and cryo-TEM results for PEG150-stearate 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.

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