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Published in:
Langmuir

DOI:
10.1021/acs.langmuir.8b03829

Published: 19/03/2019

Please cite the original version:
Shape and Phase Transitions in a PEGylated Phospholipid System

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ABSTRACT: Poly(ethylene glycol) (PEG) polymers and PEG-conjugated lipids are widely used in bioengineering and drug transport applications. A PEG layer in a drug carrier increases hydrophilic repulsion, inhibits membrane fusion and serum opsonin interactions, and prolongs the storage and circulation time. It can also change the carrier shape and have an influence on many properties related to the content release of the carrier. In this paper, we focus on the physicochemical effects of PEGylation in the lipid bilayer. We introduce laurdanC as a fluorophore for shape recognition and phase transition detection. Together with laurdanC, cryogenic transmission electron microscopy, differential scanning calorimetry, molecular dynamics simulations, and small-angle X-ray scattering/ wide-angle X-ray scattering, we acquire information of the particle/bilayer morphology and phase behavior in systems containing 1,2-dipalmitoyl-sn-glycero-3-phosphocholine:1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG(2000) with different fractions. We find that PEGylation leads to two important and potentially usable features of the system. (1) Spherical vesicles present a window of elevated chain-melting temperatures and (2) lipid packing shape-controlled liposome-to-bicelle transition. The first finding is significant for targets requiring multiple release sequences and the second enables tuning the release by composition and the PEG polymer length. Besides drug delivery systems, the findings can be used in other smart soft materials with trigger-polymers as well.

INTRODUCTION

In aqueous solutions, phospholipids form various structures. These include liposomes and micelles. Bicelles are a specific subgroup of micelles. They contain a bilayer and are discoidal in shape. Liposomes are used in drug delivery due to their ability to encapsulate hydrophilic molecules into their aqueous core. The hydrophobic part in the bilayer can also be used since lipophilic molecules have an affinity toward the lipid tails. Hence, liposomes,1 bicelles,2 and micelles3,4 can be used in drug delivery purposes alike. In the most typical scheme, a target cell internalizes a carrier via endocytosis and a hydrophilic drug is released by some internal or external trigger. These include changes in pH, temperature,6 or radiation in the ultraviolet,7 visible,9,10 or near infrared9,11–14 region. Here, the trigger is set to alter the phase order in the lipid bilayer, resulting in an increased permeation and release of the drug14,15 or any encapsulated cargo.16

Drug delivery systems face many obstacles as they pass through the hostile environments of epithelia. Unfortunately, liposomes are usually targeted by serum opsonin and opsonized liposomes are removed from the circulation by specialized phagocytic cells in a matter of hours.17,18 Surviving this clearance is a major objective, because a longer circulation time increases chances of reaching the target cell. Opsonin targeting can be prevented by adding poly(ethylene glycol) (PEG) moieties on the surface of the carrier, usually at the concentration of ca. 5 mol %, which is a procedure sometimes portrayed as “the gold standard.”19,20 The PEG layer adds a steric barrier against opsonins and other bioactive molecules.21 It increases circulation time and provides a stealth sheath that stabilizes the drug delivery system in blood and in storage. A good example of this approach is Doxil (a liposomal drug formulation that is used to deliver chemotherapeutic doxorubicin into the cancer cells). Doxil was approved by the FDA in 1995.22

In addition to the steric barrier, PEG changes the extent of hydration of the bilayer23 and inhibits its interaction with divalent ions, most commonly Ca2+, decreasing the extent of membrane fusion.24,25 Interestingly, PEG can be solubilized in both hydrophilic and hydrophobic solvents.26 Hence, it can be used to bind not only the hydrophilic molecules, like albumin,27,28 but also more hydrophobic molecules, e.g.,...
indocyanine green.\textsuperscript{12,28} Due to its hydrophobic moieties, it has been hypothesized that PEG may even partly penetrate into the liquid crystal or fluid phase of the bilayer found at high temperatures.\textsuperscript{25} This obviously affects the behavior of a lipid system as a drug carrier and releaser.

PEG has been studied experimentally and computationally. In coarse-grained molecular dynamics (MD) simulations, transferable PEG models have been introduced by Lee et al.\textsuperscript{30} Rossi et al.\textsuperscript{31} and Grunewald et al.\textsuperscript{32} These models have been utilized in simulations of PEGylated lipid bilayers and lipid aggregates.\textsuperscript{25,33–38} Experimentally, PEGylation has been shown to not only affect the phase transition behavior of the lipid systems,\textsuperscript{39} but also the particle shape. The latter was also obtained in simulations by Lee and Pastor.\textsuperscript{33}

The shape of the lipid aggregate depends on its lipids. For example, pure 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) form liposomes and pure PEG-terminated 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG) forms globular micelles, but with sufficient PEGylation they both form bicelles.\textsuperscript{40–43} Similarly, \( \alpha \)-phosphatidylcholine (EPC) forms liposomes but the EPC:DSPE-PEG system forms long cylindrical micelles instead of bicelles.\textsuperscript{40,41,43,44} The shape is usually predicted by the spontaneous curvature\textsuperscript{45} (or the packing parameter\textsuperscript{49,50} of the lipid or the lipid systems containing DPPC and DSPE-PEG with cholesterol (40 mol %) forms bicelles due to the stiffness of the bilayer.\textsuperscript{51} The rigidity of the bilayer plays a key role\textsuperscript{52} because it affects the segregation of lipids. For instance, EPC:DSPE-PEG with cholesterol (40 mol %) forms bicelles due to the stiffening of the tail groups.\textsuperscript{49,50}

In this paper, we have studied the physicochemical effects of PEGylation on the structure, shape, and sizes of the lipid systems containing DPPC and DSPE-PEG. We introduce a new method for the shape recognition using fluorescence emissions of laurdanC. This method is presented along with several other techniques, including new MD simulations that provide a pervasive view on the two effects of PEGylation. The first effect is the shift in the phase transition temperature, measured with differential scanning calorimetry (DSC) and laurdanC. This shift could be utilized in partial drug release with, e.g., phase transitions from long cylindrical micelles instead of bicelles.\textsuperscript{40,43,44} The latter was also obtained in simulations by Lee and Pastor.\textsuperscript{33}

Preparation of Lipid Samples. Samples containing laurdanC were prepared according to ref 14 but using N-(2-hydroxyethyl)pyranezine-\( \text{N} \)-ethanesulfonic acid (HEPES) buffer (150 mM NaCl, 20 mM HEPES in pH 7.4) instead of Tris–HCl. Other samples were prepared as follows: 20 \( \mu \)mol of lipids in chloroform, containing 0, 3, 6, 9, 12, 15, or 24 mol % of DSPE-PEG(2000) were added to a round flask. Chloroform was evaporated in a nitrogen stream and the remaining lipids were heated up to 60 °C prior to the addition of 2 mL of HEPES buffer. The dispersion was sonicated in a 60 °C bath sonicator for the duration of adding the buffer solution (\( \leq 45 \) s). After this, the dispersion was extruded eleven times through double-stacked polycarbonate membranes with the pore size of 200 nm. The temperature in both steps was constant, at 60 °C. After the extrusion, samples were stored in a refrigerator overnight before further studies. In cryo-TEM, Tritivated lipid samples were prepared in FEI Vitrobot (FEI) after Kuntsche et al.\textsuperscript{54} and Iancu et al.\textsuperscript{55} Briefly, ethane gas was condensed halfway into a liquid-nitrogen-cooled Vitrobot cup following liquefaction of propane gas and resulting in a liquefied gas mixture of 1:1 ethane:propane. A plasma-treated (30 s H\( _2 \)-O\( _2 \), Gatan Solarus 950, Gatan) QUANTIFOIL R 2/2 (or R 2/1) copper TEM grid (Electron Microscopy Sciences) was placed inside the Vitrobot chamber and the humidity was increased to 100% at 22 °C. 5 \( \mu \)L of the sample was pipetted onto the TEM grid, the grid was blotted with a filter paper for 1 s, and immersed into the ethane–propane mixture. The grid was then moved into the cryo-TEM sample holder under liquid nitrogen. Micrographs were taken with an FEI Tecnai T12 (FEI) transmission electron microscopy (TEM) with 120 kV accelerating voltage.

The size distribution and the number of distinctive particles were determined with a self-made Matlab script (Supporting Information). Each particle type was classified to groups of "liposomal", "bicellar edge" (bicelle edge toward the microscope), "bicellar face" (bicelle face toward the microscope), and "unknown" (irregular shapes) structures. Structures at the vicinity of 5 nm in diameter were excluded from the image analyses since they are difficult to distinguish from the background. The number fraction of bicelles \( n_{\text{bicelle}} \) \textsuperscript{58} was determined from the number of bicellar shapes vs the total number of particles. However, the signal intensity in, e.g., SAXS, DSC, and dynamic light scattering does not follow the number of particles but the number and the molar mass of monomers in the particles. The number of surfactant molecules is related to the surface area of the particles. Hence, the monomeric bicelle fraction can be determined as

\[
\frac{N_{\text{TEM}}}{N_{\text{bicelle}}} = \frac{N_{\text{bicelle}}}{N_{\text{TEM}}} + \tau N_{\text{liposome}}
\]

where \( \tau \) is the area-scaling factor. It is a function of the average area of liposomes and bicelles

\[
\tau = \frac{\langle \text{liposome} \rangle^2 + (\langle \text{liposome} \rangle - \langle \text{bicelle} \rangle)^2}{2\langle \text{bicelle} \rangle + \tau \langle \text{liposome} \rangle d + d^2}
\]

where \( d \) is the thickness of the bilayer, and \( \langle \text{liposome} \rangle \) and \( \langle \text{bicelle} \rangle \) are the average radii of the corresponding particle. Differential Scanning Calorimetry and Fluorescence Spectroscopy. DSC thermograms were measured with a MicroCal VP-DSC calorimeter (MicroCal Inc., Northampton, MA) between 10 and 60 °C, starting at 10 °C. Heating and cooling rates were 1 and 0.5 °C.
min^{-1}, respectively, to obtain similar peak positions and shapes, considering the differences in the phase separation kinetics. Both scan directions were measured twice to ensure the reversibility of the system.

Laurdan C measurements were performed with a PerkinElmer LS5 fluorescence spectrometer in a temperature controlled four-window cuvette. Each measurement was started after stabilization for 3 min at the given temperature between 25 and 70 °C. Smaller temperature intervals (0.5 °C) were used near the phase transition region (i.e., 34–45 °C). The lipid order was calculated with the laurdan generalized polarization (GP)

\[ GP = \frac{I_1 - I_3}{I_1 + I_3} \]

where \( I_1 \) and \( I_3 \) represent the emission intensities at the wavelengths corresponding to the ordered (\( \lambda_{em} = 430 \) nm) and disordered state (\( \lambda_{em} = 510 \) nm) of the lipid bilayer. In addition to GP, the derivative \( dGP/dT \) was determined to portray the data similar to the DSC data.

The deconvolution of the DSC data and \( dGP/dT \) peaks was calculated using the Voigt profile analysis package by Ruzi, using the Faddeeva function algorithm.57 The Matlab script fits multiple Voigt profiles, i.e., the convolution of Gaussian distribution and Cauchy–Lorentz distribution, simultaneously. The number of fitted Voigt profiles was set to 7 in DSC data and 5 in the GP differentials. Signals that were clearly part of the baseline, had zero intensity, or were part of the pre-phase transition were subtracted. Typically, the DSC fits found 3 distinctive Voigt profiles below the main phase transition peaks in DSC but, e.g., the PEG9 sample contained 5 distributions in the upward scan and 4 distributions in the downward scan. The peaks were labeled in the order of their position and compared with the like measurement (e.g., downward scan of PEG9 with the other downward scans).

Small/Wide-Angle X-ray Scattering. The SAXS/WAXS measurements were performed using a SAXSLab Ganesha 300XL instrument (SAXSLab ApS, Skovlunde, Denmark), a pinhole collimated system equipped with a Genix 3D X-ray source (Xenocs SA, Sassenage, France). The scattered intensity was recorded with the detector placed at three sample-to-detector distances yielding a range of the scattering vector \( q \) of 0.05–20.0 nm\(^{-1}\). The samples were sealed in a 1.5 mm diameter quartz capillary (Hilgenberg GmbH, Malsfeld, Germany), and the measurements were performed at 25, 41, and 50 °C. In all cases, the temperature was controlled with water circulation with an accuracy of 0.2 °C. The two-dimensional scattering pattern was recorded by using a two-dimensional 300 K Pilatus detector (Dectris Ltd., Baden, Switzerland) and the radial averaging to obtain \( I(q) \) was performed with SAXSGui software. The measured scattering curves were corrected for solvent scattering.

The following analyses for the SAXS data were derived following refs 58–65. The total scattering cross-sections are the sum of the lipidosome and bicelle scattering (i.e., \( I_{bicelle} \) and \( I_{liposome} \))

\[ I_{tot} = x_{bicelle}I_{bicelle} + (1 - x_{bicelle})I_{liposome} \]

where the fraction of bicelles \( x_{bicelle} \) was determined cryo-TEM. In this form, we assume no interaction between particles. The starting point of \( I_{bicelle} \) and \( I_{liposome} \) is the corresponding core--shell structure. These are detailed in Supporting Information. Briefly, the form factor of the lipidosome is

\[ P_{liposome} = \sum_{f, i} \frac{4}{3} \Delta \rho V_f \frac{\sin q r_{ij} - q r_{ij} \cos q r_{ij}}{(q r_{ij})^3} \]

where each component layer follows the Rayleigh form factor amplitude,66 \( \Delta \rho \approx \rho_{ave} - \rho \) is the scattering length contrast between the component layers, \( r_{ij} \) is the radius, and \( V_f \) is the volume of the component layer, which is deflected as a function of the polydispersity index (see the Supporting Information). For the bicelle, the form factor is

\[ P_{bicelle} = \sum_{f, i} \frac{4}{3} \Delta \rho V_f \frac{\sin q r_{ij} - q r_{ij} \cos q r_{ij}}{(q r_{ij})^3} \]

with

\[ f_{ij} = \sum_{i} \Delta \rho V_f \frac{\sin q r_{ij} - q r_{ij} \cos q r_{ij}}{q r_{ij}^3} \]

where each component layer follows the cylindrical symmetry according to Fournet,67 \( \alpha \) is the angle between the incident beam and the cylinder normal, \( f_{ij} \) is the modified Bessel function of order 1, and \( l \) is the half-thickness of the component layer. When the particle surface is covered with PEG, the total scattering cross-section is given as\(^{66,68}\)

\[ I_{tot} = n(P + N[P + 2Pc + (N - 1)Pc]) \]

where, in the order of appearance, \( P \) and \( P_c \) are the self-correlation terms of the lipid core and the PEG chain, respectively, \( P_m \) and \( P_a \) are the cross-correlation terms of the core–chain and the chain–chain interactions, respectively, \( n \) is the macroscopic number density of the particles, and \( N \) is the number of polymer chains on a single particle. Since PEG is essentially a Gaussian random coil with the characteristics detailed by Hammouda,69,70 the self-correlation term is the well-known Debye function

\[ f_{ij} = \frac{\Delta \rho_{PEG} V_{PEG}}{\Delta \rho_{PEG} V_{PEG}} \left[ \exp(-q R_{ij}) + (q R_{ij})^2 \right] \]

where \( \Delta \rho_{PEG} = \rho_{PEG} - \rho_0 \) is the scattering length density contrast of the PEG chain, \( V_{PEG} = \) the partial specific molecular volume of PEG, and \( R_{ij} \) is its radius of gyration. The form factor amplitude times \( \Delta \rho_{PEG} V_{PEG} \) is given as

\[ f_i = \Delta \rho_{PEG} V_{PEG} \left( 1 - \exp(-q R_{ij}) \right) \left( q R_{ij} \right)^2 \]

There is some irregularity in the presentation of \( f_i \) in the literature. Most authors, e.g., refs 58–60 and 65 follow Hammouda,69 but for instance, refs 61, 64 have also used \( f_i = \Delta \rho_{PEG} V_{PEG} \left[ \exp(-q R_{ij}) \right] \left( q R_{ij} \right)^2 \). The expression \( P = 2 \left( \Delta \rho_{PEG} V_{PEG} \right) \left[ \exp(-q R_{ij}) \right] \left( q R_{ij} \right)^2 \) that is found in ref 65 may also cause confusion. Since there should be a general proportionality \( P \propto f_i \), the use of eqs 8 and 9 is well justified since \( f_i \) with a Taylor approximation \( \exp(-q R_{ij}) \approx 1 - 2(q R_{ij})^2 \), results in \( P \).

The cross-correlation terms of eq 7 with no azimuth angle dependency can be obtained by combining eqs 5 and 6 with eq 9

\[ P_{bicelle} = \sum_{f, i} \frac{4}{3} \Delta \rho V_f \frac{\sin q r_{ij} - q r_{ij} \cos q r_{ij}}{(q r_{ij})^3} \]

where \( \Delta \rho \) is the form factor amplitude of an infinitely thin shell that connects the polymer with the surface of the underlying core structure.64 For a lipidosome with a PEG fraction \( x_{PEG} \) on its outer leaflet and \( x_{in} = 1 - x_{PEG} \) on its inner leaflet

\[ \Xi_{ij} = x_{PEG} \sin q (r_{ij} - R_{Cl}) + x_{in} \sin q (r_{ij} + R_{Cl}) / q(r_{ij} + R_{Cl}) \]

Since liposomes are relatively large, a good approximation is to let \( x_{PEG} \to 0.5 \). In this case, \( x_{PEG} \) and \( x_{in} \) can be omitted from eq 11 and the multipliers in eq 7 can be effectively substituted with \( N_i \to N_i / 2 \), which is the approach presented by Arleth et al.64 The form factor amplitude of a cylindrical shell is

\[ \Xi_{ij} = \sum_{f, i} \frac{2}{3} \Delta \rho V_f \frac{\sin q r_{ij} \cos(q l_{ij} + R_{Cl} \cos q l_{ij})}{q l_{ij} + R_{Cl} \cos q l_{ij}} \frac{\sin(q l_{ij} \cos q l_{ij})}{q l_{ij} \cos q l_{ij}} \]

where the former term describes the fraction \( x_{PEG} \) of the form factor amplitude with PEG only on the face of the bicelle and the latter
describes the fraction \( x_{\text{rim,uniform}} \) on the rim region. Uniform polymer coating is obtained when \( x_{\text{rim,uniform}} = 2(1 + 2\epsilon)^{-1} \).

**Computational Methods.** Molecular dynamics (MD) simulations of pure DPPC and mixtures of DPPC and DSPE-PEG(2000) in water were performed to support the experimental observations. The model used here is based on the implicit-solvent version of the CG MARTINI model,12,13 called DRY-MARTINI.13 Similar to the standard MARTINI PEG models,30,31 a 3-to-1 CG mapping for the PEG is used, whereas the lipids are treated with the 4-to-1 DRY-MARTINI CG mapping scheme. Additional model details are provided in the Supporting Information.

The simulations were run with Gromacs 5.1.6–8 in the NVT ensemble. Temperature was maintained at \( T = 323 \text{ K} \) by the stochastic velocity rescaling thermostat of Bussi et al.,9 with the stochastic friction time constant \( \tau_s = 4.0 \text{ ps} \). The equations of motion were integrated by the second-order stochastic dynamics integrator using a time step of 40 fs. A cut-off of 1.1 nm was used for both the Lennard-Jones (LJ) and electrostatic interactions with the LJ interaction potential shifted to zero at the cut-off. As for the reaction-field electrostatics, relative permittivity \( \epsilon_r = 15 \) was used beyond the cut-off distance. In total, the simulation protocol follows the standard DRY-MARTINI protocol13 augmented by the improved Verlet integration protocol of ref76.

Following the experimental setup, mixtures of DPPC and DSPE-PEG(2000), i.e., DSPE lipids with 45 CH₂CH₂O monomers, are examined. The structures and the construction of the DSPE-PEG model can be found in our earlier publications.36,73 Four different molecular compositions were studied. These contained (1) 10 500 DPPC molecules (PEG0), (2) 10 269 DPPC and 231 DSPE-PEG molecules (PEG2.2), (3) 9400 DPPC and 1100 DSPE-PEG molecules (PEG10.5), and (4) 5250 DPPC and 5250 DSPE-PEG molecules (PEG50). Each lipid was also associated with a Na⁺ counter-ion to maintain the electroneutrality in the systems. The water interaction in the system is given implicitly by the DRY-MARTINI model.

Initial configurations were constructed by distributing the lipids randomly in the simulation box. The PEG chains were set to an extended coil state. A simulation box of 50 nm × 50 nm × 50 nm was used with PEG0, PEG2.2, and PEG10.5 systems and 100 nm × 100 nm × 100 nm × 100 nm for the PEG50 system. For assessing lipid segregation and bicelle thickness, 30 nm × 30 nm × 30 nm systems of self-assembled single bicelles of size 1050 lipids, with the corresponding DSPE-PEG2000 fractions, were analyzed. All systems were initially energy minimized by using the steepest descent algorithm after which an MD run was performed in the NVT ensemble with the total duration of 0.5 μs.

**RESULTS AND DISCUSSION**

**Microscopic Characteristics of PEGylated lipid systems.** Typical cryo-TEM image of the PEG3 (A) and PEG24 (B). Scale bar is 200 nm. (C), (D), and (E) display liposomes, edge bicelles, and face bicelles, respectively. The total fraction and the subset bicelle fraction are depicted in (F) with bicelle face and edge as filled diamonds and unfilled diamonds, respectively. The size of the liposomes (dots) and bicelles (crosses) is shown in (G).

Folded bicelles (in PEG9) could be observed. The fractions of the bicellar units are shown in Figure 1F. As can be seen, bicellar units start to emerge rapidly at \( x_{\text{PEG}} > 0.09 \) and bicellar faces appear in the sample with 15 mol % of PEGylation. Thus, the system undergoes a transformation from liposomes to bicelles at ca. 10 mol % and the orientation becomes more random when the bicelle size decreases,78 which is evident from the cryo-TEM data in Figure 1G. Although the number of bicelles increases, their average size decreases and the size of the remaining liposomes increases. This marks a clear separation between the shapes and it shows that the smallest units have a strong affinity to produce disks.

The SAXS patterns (Figure 2A) of the low PEGylation resembles those of Pili et al.,79 who measured similar systems with synchrotron-based SAXS. The SAXS data were fitted (red lines) with parameters in Table S1 of the Supporting Information. The particle radius (Figure 2B) was determined with longer lipids (i.e., DSPE-PEG). The radii match well with the cryo-TEM results; black line in Figure 2B shows the average radii from Figure 1G.

The radius of gyration \( R_G \) showed a gradual increase (Figure 2D) when PEG was added. With low PEGylation (PEG3), the effect of \( R_G \) is very small and more accurate estimation is difficult to obtain. At high PEGylation, \( R_G \) goes to 1.75 nm and increases to 1.92 nm in samples containing only bicelles. These values are reasonable for PEG with a molar mass of 2 kDa.80,81

The location of the polymers was also determined. On PEG3 liposomes, the PEG moieties have no preference, but in PEG6, the proportion (31:69) favors the outer leaflet. In bicelles, the PEG units are more readily found on the edge, as can be seen in Figure 2E, where the ordinate displays the difference between the fitted value and the uniform polymer coating (\( x_{\text{rim,uniform}} = 2(1 + 2\epsilon)^{-1} \)).

The fitting parameters differ slightly from those in the literature. The SANS data of Arleth et al.64 is equivalent to \( \rho_{\text{PEG}} = 1.11 \times 10^{11} \text{ cm}^{-2} \), \( \rho_{\text{tail}} = 0.83 \times 10^{10} \text{ cm}^{-2} \), and \( \rho_{\text{head}} = 1.45 \times 10^{12} \text{ cm}^{-2} \). Quite close to this, we obtained \( \rho_{\text{PEG}} = 1.08 \times 10^{12} \text{ cm}^{-2} \) and \( \rho_{\text{tail}} = 0.87 \times 10^{11} \text{ cm}^{-2} \) for liposomes and \( \rho_{\text{tail}} = 0.85 \times 10^{11} \text{ cm}^{-2} \) for bicelles. The head group scattering length densities had more variation, stemming partially from the difference of D₂O buffer and water. This is somewhat
expected; Pedersen and Gerstenberg have shown that SAXS measurements of polymer micelles in H2O cannot be readily fitted with parameters obtained from SANS measurements in D2O.

The SAXS fits in Figure 2A are accurate in the q range 0.1–2 nm−1 for all PEGylated samples, and the fits of PEG12, PEG15, and PEG24 are accurate in the entire lower q range. The intermediate range provides the shape and size of the particles. Thus, the small divergence of the PEG3 and PEG6 fits in the very low q range is not significant, and the presence of spherical and discoid structures is consolidated with SAXS. The radii (Figure 2C) are almost equal to those measured with cryo-TEM (Figure 1G) although the radius of the PEG24 bicelle was smaller than that in the cryo-TEM images. This may result from the appearance of particles smaller than the ones quantified in cryo-TEM.

The fits in Figure 2A deviate from the measurements in the high q range. For simplicity, the fitting function did not contain a constant effective background to account for scattering associated with the internal fluid structure of the bilayer. Thus, the fit decays faster at high q than the experimental data. Nevertheless, the fit clearly captures the form factor oscillations, proving that the thicknesses in Figure 2C are in the correct size range. Yet, the determination of the bilayer thickness may not be accurate because the high q region is susceptible to errors in solvent subtraction and the measured intensities do not fall between the behavior of a centrosymmetric sphere model (q−4) and a Gaussian random coil model (q−2) in either the liposome or bicelle system.

The shape transformation was also verified with MD simulations in Figure 3. Figure 3A shows pure DPPC, panel (B) DPPC with 2.2 mol % DSPE-PEG, panel (C) DPPC and 10.5 mol % DSPE-PEG, and panel (D) 50 mol % PEGylated lipid. Panel (E) shows a detailed structure of a bicelle of 1050 molecules containing 10.5 mol % DSPE-PEG, and panel (F) is the DSPE lipid density profile of the same bicelle.

Figure 2. A) SAXS fits (red lines). Black dots represent the SAXS measurements of PEG3 (k = −2), PEG6 (k = −1), PEG12 (k = 0), PEG15 (k = 1), and PEG24 (k = 2). Fit parameters for liposomes (dots) and bicelles (crosses): (B) radius (the line is the cryo-TEM average), (C) bilayer thickness, (D) radius of gyration of PEG, and (E) excess amount of PEG on the rim region.

Figure 3. Snapshots of the aggregates in 0.5 μs molecular dynamics simulations. Panel (A) shows pure DPPC, panel (B) DPPC with 2.2 mol % DSPE-PEG, panel (C) DPPC and 10.5 mol % DSPE-PEG, and panel (D) 50 mol % PEGylated lipid. Panel (E) shows a detailed structure of a bicelle of 1050 molecules containing 10.5 mol % DSPE-PEG, and panel (F) is the DSPE lipid density profile of the same bicelle.
The elongated micelles. Thus, the transition observed in the molecular modeling is in very good agreement with the cryo-TEM and SAXS results. In addition to larger simulations, Figure 3E shows a PEG10.5 bicelle of 1050 molecules. Here, the PEGylated lipids prefer the edge more likely than the bicelle face, in accordance with the SAXS result in Figure 2E and earlier computational works by Lee and Pastor and Shinoda et al., who showed that the translocation decreases the line tension at the bicelle edge. This effect is also depicted in Figure 3F, where the density profile of DSPE lipids in bicelle (Figure 3E) is presented. These simulations represent systems with ca. 140 mM of lipids, which is 14-fold the experimental value in the equilibrium but realistic as a local concentration during the self-assembly. The concentration should not have a significant effect on the aggregate shape, because it is mainly controlled by molecular packing.

The simulations in Figure 3A–D contain 10,500 lipids. Due to the system size and finite simulation time, the formed aggregates are smaller than in the experiments. For instance, the 2.2 mol % DSPE-PEG system (Figure 3B) produces liposomes with a mean radius of 7.5 nm, whereas cryo-TEM (Figure 1H) and SAXS analysis (Figure 2B) result in a radius closer to 35 nm. On the other hand, the simulation with 10.5 mol % DSPE-PEG (Figure 3C) leads to the formation of a set of bicelles with the largest ones between 9.5 and 11.5 nm in radius. These values are close to those obtained in the experiments. The PEG chain radius of gyration was 1.54 nm in all simulated samples. The value is in good agreement with the hydration shell. Finally, in the simulations, the thickness of the bilayer corresponding to PEG3 is 5.0 ± 0.1 nm and increases for PEG11.1 to 5.2 ± 0.5 nm (see the Supporting Information). This trend is in line with the SAXS results, even though the SAXS fits are less sufficient in the high $q$ region and the fitted head group is more involved with the water barrier than the simulations.

Thermal Analyses and the Phase Transition in the Lipid Bilayer.

The structures of lipid bilayers showed no significant differences in the WAXS measurements of PEG3, PEG12, and PEG24 (Figure 4A, up-to-down), or in the samples shown in Figure S1. The lipid bilayer portrays near hexagonal packing or gel phase at 25 °C since the Bragg peak is found at ca. 14.5 nm $^{-1}$ (blue line). At 50 °C (orange line), the bilayer is in the fluid phase and only a broad peak is observed just below 14 nm $^{-1}$. At 41 °C (red line), DPPC is in the ripple phase and the Bragg peak is found at ca. 14.4 nm $^{-1}$. However, the overall shape is almost equal to that at 25 °C.

The downward DSC scan of a PEG12 sample and the corresponding laurdanC generalized polarization differential are shown in Figure 4B. As can be seen, the enthalpy can be deconvoluted to four contributions. Similarly, GP differentials (Figure 4C), downward (Figure 4D), and upward (Figure 4E) DSC peaks are composed of multiple peaks. The emergence of particulate subgroups stems mainly from the uneven PEGylation. The average melting temperature increases as a function of PEG fraction. This can be expected since pure DPPC bilayers melt at ca. 41 °C, whereas a DSPE bilayer undergoes a phase transition at ca. 74 °C. The increase in the phase transition temperature is also connected with the decrease in the overall lateral pressure as the fatty acid chains of DPPC and DSPE-PEG become increasingly mismatched as the membrane enriches with DSPE-PEG. In addition to the increase in the melting temperature as shown in Figure 4,
imply that the onset of bicellation takes place between 9 and 12 mol % of PEG in the formulation. According to the laurdanC measurements, this transition is quite sharp. Comparison of xbicelle (calculated from Figure 4C) with the logarithm of xbicelle, shows a change in the slope at xPEG = 0.096 (see Figure 6A,B). We interpret this point as the critical bicellation fraction (CBF).

LaurdanC measures electric polarity in its proximity.88,89 Hence, its response in the bilayer is related to the area of the lipid–water boundary.90,91 In the preliminary MD simulations, we found that laurdanC has lower mobility in the bilayer than the regular laurdan molecule because of its way of making hydrogen bonds with the lipid headgroups. This leads to assume that laurdanC resides in the facet of the bicelle rather than in the rims. This assumption is also supported by the fact that fraction xbicelle does not approach unity when xbicelle → 1 (Figure 6A). Hence, we can derive an equation for the laurdanC GP fraction

\[
x_{\text{LC}} = \frac{A_{\text{flat}}}{A_{\text{flat}} + A_{\text{curved}}} = \frac{2(\eta - 1)^2}{2(\eta - 1)^2 + \pi(\eta - 1) + 1} \tag{13}
\]

where \(A_{\text{flat}}\) and \(A_{\text{curved}}\) are the total areas of the flat and curved surfaces and \(\eta = 1 + \frac{R_{\text{fl}}}{d_{\text{HH}}}\) is the aspect ratio. Here, the shape of the bicelle is the same as presented by Bergström,92 with \(R_{\text{fl}}\) being the radius of the facet and \(d_{\text{HH}} = 4.4 \text{ nm}\) being the approximate bilayer thickness from the lipid head group to another. When the measured xbicelle are used to calculate \(\eta_{\text{LC}}\) with eq 13, an excellent correlation with \(\eta_{\text{TEM}}\) (determined from the micrographs) is obtained as shown in Figure 6C. LaurdanC measurement can thus determine the shape (aspect ratio) of the lipid system directly.

The limit radius \(R_{\text{flat limit}} = d_{\text{HH}}(\eta_{\text{limit}} - 1)\) of bicelles can be obtained by calculating the aspect ratio of the limit xbicelle → 0.684 (Figure 6A) with eq 13. With \(\eta_{\text{LC}}\), this radius limit goes to R_{\text{flat limit}} = 16.2 nm, which is presumably the hydrodynamic

### Table 1. Fraction of Bicelles in DSC Samples xbicelle Compared to Cryo-TEM (xbicelle) and the Molar Enthalpy Change ΔHliposome and ΔHbicelle

<table>
<thead>
<tr>
<th>xPEG</th>
<th>x_{DSC} bicelle (x_{TEM} bicelle) (%)</th>
<th>ΔHliposome (kJ mol⁻¹)</th>
<th>ΔHbicelle (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.09</td>
<td>1.72 0.12 0.15 0.24</td>
<td>29.49 12.51</td>
<td></td>
</tr>
<tr>
<td>DSC up</td>
<td>19.97 45.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSC down</td>
<td>30.61 47.82</td>
<td>83.93 13.82</td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>17.38 0.01 43.97 0.85</td>
<td>83.44 5.96</td>
<td></td>
</tr>
</tbody>
</table>

4D,E, broad phase transition peaks, extending below 41 °C, are obtained in the liposomal samples. This is caused by the existence of smaller liposomes (<80 nm in diameter) with wide size distribution.87

The DSC deconvolution peaks were also color coded as displayed in Figure 4D,E. In the case of laurdanC measurements (Figure 4C), only liposomal (blue) and bicellar (red) phase transition peaks were extracted because the signal contains much less data points than the DSC measurements. Both the DSC upward and downward scans of the entire sample set exhibited ten deconvolution peaks. The phase transition peaks were extracted because the signal magnitude, broad phase transition peaks, extending below 41 °C, are noticeably decreased in the average enthalpy values. The enthalpies in Table 1 represent only the main phase transition; the pre-phase transition was eliminated from the Table 1 set exhibited ten deconvolution peaks. The phase transition temperature is observed in the PEG-rich samples, the melting process is not completely reversible. Instead, a shift toward higher phase transition temperatures is observed in the PEG-rich samples. Both the DSC upward and downward scans of the entire sample set exhibited ten deconvolution peaks. The phase transition temperatures are obviously not the same because the kinetics of freezing and melting are slightly different.

Figure 5A shows the deconvoluted subgroup peaks from Figure 4D,E with indexes from 0 to 9 in the order of their phase transition temperature. The error bars in Figure 5A mark the 95% confidence interval. The average structure in each sample is shown in Figure 5B. It shows that when the samples are kept for 30 min at 60 °C, the melting–freezing process is different. Instead, a shift toward higher phase transition temperatures is observed in the PEG-rich samples, proving again that liposome structures in PEG12 and PEG15 are less stable than bicellar structures. The bicelle fraction was also calculated as x_{bicelle} = ΔH_{bicelle}/ΔH_{tot} where the enthalpy of bicelles was calculated as the sum of the enthalpies of structures 8 and 9. This value is rather similar to that obtained with cryo-TEM (Table 1). In addition, the molar enthalpy change ΔH_{bicelle} and ΔH_{liposome} were calculated using x_{bicelle} values.

The enthalpies in Table 1 represent only the main phase transition; the pre-phase transition was eliminated from the deconvolution for the sake of simplicity. Hence, liposome enthalpies are ca. 2–4 kJ mol⁻¹ lower than in the raw data. Table 1 shows a noticeable decrease in the average enthalpy when the system transforms from spherical liposomes to discoid bicelles. Effect of Spontaneous Curvature. Preceding chapters imply that the onset of bicellation takes place between 9 and 12 mol % of PEG in the formulation. According to the laurdanC measurements, this transition is quite sharp. Comparison of x_{bicelle} (calculated from Figure 4C) with the logarithm of x_{bicelle} shows a change in the slope at x_{PEG} = 0.096 (see Figure 6A,B). We interpret this point as the critical bicellation fraction (CBF).

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radius. When the $\eta_{LC}$ values are corrected to the TEM aspect ratio scale with the trend line in Figure 6C, an aspect ratio limit of 4.69 is reached (red line in Figure 7A), that is equivalent with the disc radius $R_{flat}$ limit $TEM = 13.7$ nm (red line in Figure 7B).

With the two critical parameters, CBF (or $x_{PEG}$) and $R_{flat}$ limit $TEM$, we can also set an empirical relationship for the bicelle radii as

$$f = \frac{1}{2}\kappa(K_{12} - K_0)^2 + \gamma$$

Even though the terms $\kappa l$ and $K_G$ are of the same magnitude in eq 15, the latter term has little effect on the shape ($K_G$ is more important when membrane fusion and fission are in question). Hence, the Gaussian curvature contribution can be omitted. In addition, it should be noted that the spontaneous total curvature $K_0$ is double of the commonly used spontaneous curvature with the usual notations $c_0$ or $H_0$. The minimum of eq 16 is the minimum of the free energy and therefore one can obtain a prediction for the shape transformation (see, e.g., ref 37).

Let us assume that the spontaneous total curvature of the mixture $K_{0,mix}$ follows simple additivity,

$$K_{0,mix} = x_{PEG}K_{0,DSPE-PEG} + (1 - x_{PEG})K_{0,DPPC}$$

and the surface tension is a constant. The transition occurs when the spontaneous total curvature matches the curvature $K_{12} = 1/d_{HH}$. We have used the value $d_{HH} = 4.4(5)$ to minimize eq 16 at $x_{PEG} = 0.096$. The spontaneous total curvature values of individual lipids, $K_{0,DPPC} = 0.136$ nm$^{-1}$ and $K_{0,DSPE-PEG} = 1.06$ nm$^{-1}$, can be extrapolated from the particle curvature vs particle radius plot, as shown in Figure 8A, where the lipid spontaneous curvatures are shown as black lines. The transition region is illustrated in Figure 8B, where the form of eq 16 is added to the spontaneous total curvature of the
Ashok et al., who have reported a value of 85 mol % at the takes place at ca. 84 mol %. This is in perfect agreement with making bicelle edges with curvature exist at the PEGylation fraction below bicellation. This is the most prominent phenomenon of the increased PEG fraction, we also found that other kinds of lipid particulates is important. In this paper, the effect of PEGylation on the physicochemical properties of lipid particulates is studied with cryo-TEM, DSC, SAXS, and fluorescence spectroscopy of laurdanC, as well as with molecular dynamics simulations. It was found that increasing the degree of PEGylation alters the fluidity of the bilayer and the shape of the lipid particulates. As demonstrated here, the shape-shift can be monitored with laurdanC. With the combination of laurdanC emission spectra and cryo-TEM, it was shown that the most visible transformation was from liposomes to bicelles when the fraction of the DSPE-PEG(2000) lipids reached the critical bicellation fraction (CBF), 9.6 mol %. We identified the greatest contribution to be the spontaneous curvature of the lipid components that is linked with the steric repulsion between PEG units in the lipid mixture. This means that lipids must relocate themselves to a region of curvature that matches their own.

The lipid curvature sets a limit to the sizes of units in the system. Liposome units have a limit size of ca. 30 nm which is characteristic to DPPC. Bicelle is characterized by the DSPE-PEG lipid that has an equivalent curvature of a micelle core 3.8 nm. Bicelles are formed when the lipid mixture is capable of making bicelle edges with curvature \( K_{12} = 1/d_{HH} \). The bicelle radius, in this case, was also effectively limited to the radius of ca. 14 nm.

Although the transformation to bicelles from liposomes is the most prominent phenomenon of the increased PEG fraction, we also found that other kinds of lipid particulates exist at the PEGylation fraction below bicellation. This is evidently from the phase transition peak shifts of both DSC and laurdanC. Each melting temperature represents an individual PEG-layered lipid particulate. The driving force of the increasing melting temperature comes from the PEGylated lipids that were shown to affect membrane thickness and decrease the effective lateral pressure prior to bicellation.

The shift in the phase transition temperatures of spherical vesicles is connected to an increased bilayer thickness and improved shielding due to the PEG layer that evidently changes the surface pressure of the bilayer. This effect could be used to improve drug delivery systems. Since drug release occurs most effectively at the main phase transition temperature of the lipid, a mixture of liposomes with a range of phase transition temperatures can enable partial drug release by an accurate temperature control. This can be achieved with, e.g., photothermal agents. Liposomes transform into bicelles when the spontaneous curvature of the mixture starts to favor the latter shape. The mechanism of action is a simple one. A lipid with a sizable polymer moiety on its head group has a positive curvature that controls the transformation. Hence, with a suitable polymer, it could be possible to trigger the transformation and activate the drug release.

**CONCLUSIONS**

PEGylation is the current state-of-the-art method to prolong circulation times of liposomal drug delivery systems in blood and in storage. Therefore, understanding the effects of PEGylation on the physicochemical properties of lipid particulates is important. In this paper, the effect of PEGylation in DPPC:DSPE-PEG(2000) systems was studied with cryo-TEM, DSC, SAXS, and fluorescence spectroscopy of laurdanC, as well as with molecular dynamics simulations. It was found that increasing the degree of PEGylation alters the fluidity of the bilayer and the shape of the lipid particulates. As demonstrated here, the shape-shift can be monitored with laurdanC. With the combination of laurdanC emission spectra and cryo-TEM, it was shown that the most visible transformation was from liposomes to bicelles when the fraction of the DSPE-PEG(2000) lipids reached the critical bicellation fraction (CBF), 9.6 mol %. We identified the greatest contribution to be the spontaneous curvature of the lipid components that is linked with the steric repulsion between PEG units in the lipid mixture. This means that lipids must relocate themselves to a region of curvature that matches their own.

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TEKES is acknowledged for funding via the “Light Activated Drug Delivery Systems” project (LADDS project). Academy of Finland is acknowledged for funding via grant 309324 (M.S.). Dr Dat Pham is acknowledged for the help in the DSC measurements. L.V. wishes to thank the Swedish-Finnish Cultural Foundation for a seven-day visiting grant to Lund University. This work made use of the Aalto University Nanomicroscopy Center (Aalto-NMC) premises. Computational resources from the CSC IT Center for Science, Finland, and RAMI RawMatTERS Finland Infrastructure are gratefully acknowledged.

**ADDITIONAL NOTES**

“From here on, the number after “PEG” indicates its mol % in the formulation.

bBending modulus is replaced with $\kappa \rightarrow 2000$ nm to better illustrate the parabolic minimum.

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