Title: A novel lipid-polymer system with unique properties has potential in drug delivery and biotechnology applications

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Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; C_8E_5, n-octylpentaoxyethylene; NBD-DMPE (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt)); ^{31}P-NMR, phosphorus nuclear magnetic resonance spectroscopy; D_2O, deuterium oxide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-([3-Cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

Abstract

The utility of detergent micelle and bicelle systems has been demonstrated to be a valuable tool for the study of membrane protein interactions and in structural studies. Bicelles are distinguished from micelles in that they contain a lipid bilayer that mimics the plasma membrane of cells making it more native-like than its detergent micelle counter-part. Bicelles are typically comprised of a long-chain phospholipid such as 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and either a short-chain phospholipid, typically 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), or a bile-salt derivative such as CHAPS or CHAPSO. In solution DMPC and DHPC bicelles assume a discoidal structure comprised of a heterogeneous arrangement where the short-chain lipids gather around the rim of the disk and the long-chain lipids form the flat, bilayer region of the bicelle. Aside from DHPC, CHAPS and CHAPSO few other detergents have reportedly been investigated for their ability to form bicelles with DMPC. In this study, the detergent, C_8E_5, was used to prepare mixtures with DMPC to determine if it adopts properties similar to DMPC-DHPC bicelles. Mixtures were evaluated using sedimentation equilibrium, ^{31}P-phosphorus NMR, and light scattering and compared to DMPC-DHPC bicelles. Interestingly, mixtures of DMPC and C_8E_5 assumed a spherical-shaped micellar structure, not the predicted discoidal shape. DMPC-C_8E_5 mixtures retain interesting properties rendering them particularly advantageous in studies of membrane protein interactions and hold promise as vehicles for drug delivery.

Keywords: bicelles; micelles; lipids; sedimentation equilibrium; density matching; light scattering

1. Introduction

It has been well-established that 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), a naturally derived phospholipid with a 14-carbon chain length and a choline head group, spontaneously assembles into discoidal lipid structures called bicelles when mixed with short-chain phospholipids such as 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), also naturally derived but containing a 6-carbon chain length with a choline head group [1]. These bilayered structures can also be prepared using DMPC and bile salt detergents such as CHAPS or CHAPSO in lieu of a short-chain phospholipid [1-3]. DMPC-DHPC bicelles are among the most extensively characterized bicellar structures, and they have been used successfully as membrane mimics in solution NMR studies of membrane protein structure [3,6]. These structures can be tailored to different applications by adjusting the type of lipid used. For example, the lipid tail length and the ratio of DMPC to DHPC, called the ***q*** – value, can affect the resulting physical properties of bicelles. Anionic lipids can also be included to introduce a negative charge at the bicellar surface [7]. Collectively, these properties affect the thickness and the planar length of the bicellar structure [8]. Bicelles are especially interesting for membrane protein studies because, unlike their detergent counterpart, they contain a bilayered planar region, which captures a true membrane-like environment [3,4]. Membrane protein structure and function is dependent on the surrounding detergent or lipid environment, therefore, choosing the optimal detergent or lipid system is critical and usually necessitates a screening of potential candidates due to the fact that not all systems are universally adaptable to all membrane proteins [9,12]. In this study, we present a novel detergent-lipid aggregate system prepared with DMPC, a long-chain phospholipid, and a polymer-based detergent system, C_8E_5. We compared and contrasted this system with the DMPC-DHPC bicellar system to determine if some of the important properties have been retained in this new system. We highlight several advantages of this new

detergent/lipid system, particularly in the study of membrane protein interactions and vehicles for drug delivery.

Sedimentation equilibrium (analytical ultracentrifugation) is a method that can be used to characterize the oligomeric state of membrane proteins based on a technique called density matching. In this technique the density of a buffered solution is matched to a lipid aggregate or detergent micellar solution using one of three density modifiers: D_2O, glycerol, or sucrose. The amount of the density modifier required is dependent on the choice of detergent or lipids used as well as the composition for mixed micellar systems. This approach is especially useful in studies of membrane protein oligomerization where direct knowledge of detergent binding is lacking [13,14]. Detergent micelles have been successfully density-matched in sedimentation equilibrium experiments using the density modifier, deuterium oxide (D_2O) and to our knowledge this has not been done comprehensively for DMPC-DHPC bicelles until now using three biocompatible density modifiers; D_2O, glycerol and sucrose [15]. In practice certain lipid aggregates or mixed micellar systems can give rise to complexes with very high densities making density matching near impossible without using expensive reagents, *i.e.* D_2O[18], or the addition of large quantities of density modifiers, which can be susceptible to gradient formation under prolonged periods of centrifugation [16]. We sought to find an alternative to DMPC-DHPC bicelles and other mixed micellar systems, for example, the DDM/ CHAPS/ CHS (n-dodecyl-β-D-maltopyranoside/ 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate/ cholesteryl hemisuccinate) mixed micelle system, which has reportedly been used for the preparation and purification of the human adenosine A_{2A} receptor from Baker's yeast, *Saccharomyces cerevisiae* [17,19]. Due to the presence of the maltose headgroup on DDM in this reported mixed micelle system, we predict a lipid aggregate comprised entirely of DDM to have a specific volume of approximately 0.806 cm^3 / g arising from the constituent molecular component densities of 0.622 cm^3 / g from glucose (maltose is a disaccharide of two glucose units) and 0.990 cm^3 / g arising from the reported

specific volume of the 12-carbon lipid tail (Figure 1B). These values can be obtained from the method described by Durchschlag et al. and in actuality these values vary depending on the organization and assembly of the lipid aggregate [20, 21]. Although these values are only estimates, they are sufficiently valid for our study. In the DMPC-DHPC bicelle system, we found that DHPC imparts a substantial density to the entire bicellar aggregate necessitating the use of density modifiers. Using the density matching approach in studies of membrane protein interactions, the contribution of the lipid aggregate to the measured buoyant molecular weight of an incorporated membrane protein is negated and the oligomeric state of the target membrane protein can be determined. We sought to investigate the physical properties of a novel lipid aggregate system with a lower density, yet preserving the bilayer properties of the bicellar mixtures using DMPC and the detergent, *n*-octylpentaoxyethylene (C_8E_5). C_8E_5 has a reported specific volume of 0.993 cm^3 / g [22]. C_8E_5 has been used to study membrane protein interactions in the absence of DMPC in sedimentation equilibrium experiments. With a partial specific volume (inverse density) substantially similar to water, C_8E_5 requires little need for density matching [13]. In the following study, DMPC-C_8E_5 mixtures were prepared and investigated and their physical properties compared to DMPC-DHPC bicelles. The size and shape of these lipid-detergent structures was also investigated using the method of Mazer and co-workers 1980, and later used by Glover and co-workers to evaluate the structure of DMPC-DHPC bicelles [3,23]. This approach along with analysis by light scattering was used to predict the shape of DMPC-C_8E_5 lipid-detergent aggregates to determine if they assume a discoidal arrangement of lipids and detergent molecules similar to DMPC-DHPC bicelles. We found that interestingly, these lipid aggregates assume a shape that is closer to spherical than to discoidal and the properties we observe for DMPC-DHPC bicelles are not preserved in DMPC-C_8E_5 aggregates. In and of themselves, DMPC-C_8E_5 aggregates have very interesting properties and warrant further study for their potential as a platform in membrane protein analysis and as a vehicle for drug delivery.

2. Materials and methods

2.1. Chemicals and reagents

DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and NBD-DMPE (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt)) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). C_8E_5 (*n*-octylpentaoxyethylene) was purchased from Bachem (King of Prussia, PA, USA). All lipids and detergents were used without further purification. NBD-DMPE was incorporated in small molar quantities so that absorption optics could be used to analyze the lipid aggregates for sedimentation equilibrium experiments. HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) was purchased from EMD Millipore (Billerica, MA USA) and NaCl (sodium chloride) was purchased from Avantor (Center Valley, PA, USA). D_2O (deuterium oxide) was purchased from Cambridge Isotopes (Tewksbury, MA, USA). Milli-Q water (Millipore) was used throughout all experiments.

2.2. Preparation of sedimentation equilibrium samples

2.2.1 DMPC-DHPC bicelles

All sedimentation equilibrium experiments were performed at 25 °C using a Beckman XL-A analytical ultracentrifuge. To follow the sedimentation of bicelles using absorbance optics, an NBD-labeled phospholipid probe was incorporated in a trace amount, 1:10,000 mole ratio of NBD-PE to DMPC, which was sufficient to provide an optical signal in the instrument. To 3.58 mg of lyophilized DMPC / NBD-DMPE 300 µL of buffer containing 10 mM HEPES pH 7.4, 100 mM NaCl was added in various amounts of D_2O (0, 10, 25, 40, 50 , 60, 70, 80, 90% (v/v)), glycerol (0, 2, 5, 10, 15, 20, 25, 30, 35% (v/v)) or sucrose (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 M). Next, the samples were vigorously vortexed until a homogeneous milk-like suspension was obtained. 13.03 µL of 25% (w/w) dihexanol-3-glycerophosphatidylcholine (DHPC) stock solution was added to the DMPC to a final ***q*** **-** value of 1.0. After adding the DHPC, the solution became clear. The samples were loaded into a 6-channel charcoal-filled epon centerpiece (pathlength 1.2 cm) using a reference solution containing D_2O, H_2O, and

buffer only. A volume of 120 µL of sample was loaded per channel, and the cells were loaded in a Beckman Ti-60 4-hole rotor. Equilibrium absorbance measurements (464 nm) were collected from 10,000 rpm to 35,000 rpm stepping up in speed in 1,000 rpm increments. Data was collected at each rotor speed. Initial equilibration was carried out at 10,000 rpm for 24 hours and subsequently 4 hours at each speed thereafter. A plot of the natural log of the absorbance versus the square of the radius was generated for each density modifier used. The plot was then fitted to a linear function and the buoyant molecular weight (M-*effective*), M_{eff}, was extracted from the slope. For each concentration M_{eff} was averaged over all speeds. A total of nine data points were generated for each buffer additive (D_2O, glycerol, sucrose). The densities of the bicellar solutions were measured using a Kyoto Electronics Density/Specific Gravity Meter (model# DA-210).

2.2.2. C_8E_5 mixtures

Sedimentation equilibrium data was collected for DMPC-C_8E_5 lipid-detergent aggregates at a theoretical ***q*** - value of 0.25 and a total lipid concentration of 5% (w/w). A total of nine samples were prepared at D_2O concentrations of 0.00, 3.25, 6.50, 9.75, 13.00, 16.25, 19.50, 22.75, and 26.00% D_2O (v/v). Sedimentation profiles were generated for all nine samples at rotor speeds of 15,000 rpm (18,112 x g), 20,000 rpm (32,198 x g), 25,000 rpm (50,310 x g) and 30,000 rpm (72,446 x g). The sedimentation profiles were mathematically treated using the Lamm equation and the effective molecular weight, M_{eff}, was calculated for each sample at each D_2O concentration. M_{eff} was plotted against the percentage D_2O concentration to evaluate the D_2O required to density match the DMPC-C_8E_5 or produce $M_{eff} = 0$.

2.3. Sedimentation equilibrium (AUC) density matching experiments

Three DMPC / C_8E_5 lipid solutions were evaluated at three theoretical ***q*** - values: 0.1, 0.25, and 0.5 and a total lipid composition of 5% (w/w). A small amount of NBD-labeled DMPE was

incorporated into DMPC / C_8E_5 samples, which enabled the lipid aggregates to be monitored using absorption optics. The ratio of NBD-labeled lipid to unlabeled lipid was kept sufficiently low so that the label did not influence the physical properties of the aggregates (1:500 mole ratio of NBD-DMPE:DMPC), but high enough that an appreciable absorbance signal could be acquired. Three samples were prepared at theoretical ***q*** - values of 0.10, 0.25 and 0.50 and a total lipid-detergent concentration of 5% (w/w). To each of three separate 1.5 mL Eppendorf tubes a specified volume of 50 mg / mL DMPC stock solution in prepared in chloroform was added to deliver a quantity consistent with the targeted ***q*** - value. A small amount of 66 µg / µL of NBD-DMPE chloroform solution was immediately added to deliver a 1:500 mole ratio of fluorescent-labeled lipid to unlabeled lipid. The samples were briefly vortexed and spun in a microfuge. 500 µL of Millipore water was added immediately before freezing the samples in liquid nitrogen. The samples were lyophilized to a powder. To each lyophilized sample an appropriate amount of Millipore water was added followed by a 40X concentrated stock solution of buffer resulting in a final concentration of 10 mM HEPES pH 7.4, 100 mM NaCl. The three samples were vortexed to a milky whitish yellow suspension and C_8E_5 detergent was added to each sample in quantities that would assume ***q*** - values of 0.10, 0.25, and 0.50, respectively. The samples were vortexed for several seconds to clarity. Three reference samples were prepared to a final volume of 150 µL according to the following: To three 1.5 mL Eppendorf tubes was added 146.0 µL of Millipore water followed by 40X concentrated buffer solution to a final composition of 10 mM HEPES pH 7.4, 100 mM NaCl.

Samples were loaded into a 6-channel equilibrium charcoal-filled epon centerpiece with a pathlength of 1.2 cm. 120 µL of each sample and reference was loaded in the appropriate channels. All sedimentation equilibrium studies were carried out on a Beckman XL-A analytical ultracentrifuge at 25 °C using a four-hole An-Ti 60 rotor. Absorbance spectroscopy was used to monitor the sedimentation of the lipid-detergent aggregates down the solution column at a wavelength of 464 nm corresponding to the absorbance of the NBD-labeled lipid. Samples were initially equilibrated at 10,000 rpm (8,050 x g) for 16 hours to allow equilibrium to be reached. The

point of equilibrium was evaluated at each speed using Match in the program, *HeteroAnalysis* (University of Connecticut, Storrs CT). Thereafter speeds were increased by 5,000 rpm up to 30,000 rpm (72,446 x g), and samples were left to equilibrate for 6 hours at each speed before acquiring data. Data was collected at four rotor speeds: 15,000 rpm (18,112 x g), 20,000 (32,198 x g), 25,000 rpm (50,310 x g), and 30,000 rpm (72,446 x g). The sedimentation equilibrium profiles were modeled using the Lamm equation to evaluate M_{eff} of the lipid-detergent aggregates in each of the three samples:

$$\ln(c_r) = \frac{M(1 - v\rho)\omega^2}{2RT} r^2 \qquad \textit{Eq. 1}$$

where c_r is the concentration of NBD-DMPE at radius r; M is the calculated molecular weight of the lipid-detergent aggregate, v is the partial specific volume of the lipid-detergent aggregate which was measured using a Kyoto density meter, ρ is the density of the HEPES buffer containing NaCl pH 7.4, which was also measured using the Kyoto density meter. ω is the angular velocity of the rotor, r is the radius from the center axis of rotation, R is the Boltzmann constant and T is the temperature in Kelvin.

2.4 Samples for ^{31}P-NMR analysis

Samples with a 25% (w/w) total lipid composition were prepared for ^{31}P-phosphorus NMR experiments. Each sample was prepared at a theoretical ***q*** = 0.5 on a 990 µL scale. To two separate 1.5 mL Eppendorf tubes DMPC was added to a final concentration of 158 mM. To both samples 81.45 µL of Millipore water was added followed by 99.0 µL of D_2O (10% (v/v)) and 6.6 µL of 3.0 M sodium acetate pH 5.6 buffer. The samples were vortexed to a milky, white suspension. Next, 555.44 µL of a 25% (w/w) DHPC solution was added to one sample and 222.48 µL of a 50% C_8E_5 (w/w) solution was added to the second sample in accordance with a ***q*** – value of 0.5. The samples were vortexed to clarity. Praseodymium (III) chloride powder was added to

a final concentration of 197 mM as per Glover et. al [3]. This concentration was previously determined to saturate the lanthanide-phospholipid interaction making the DMPC and DHPC ^{31}P-phosphorus signals distinguishable from one another in the NMR [24].

NMR experiments were carried out on a Bruker DRX500 spectrometer equipped with a BBI probe. One dimensional ^{31}P-phosphorus NMR spectra were recorded at 25 and 37 °C using a proton-decoupled single-pulse experiment with a minimum of 16 scans and a 100 ppm sweep width. The experiments were processed in TopSpin v. 1.3 (Bruker Corporation).

2.5 Samples for light scattering experiments

Mixtures of DMPC-C_8E_5 were prepared according to the theoretical ***q*** - value used to characterize DMPC-DHPC bicelles. The value ***q*** is determined by the mole ratio of DMPC to DHPC according to the following equation:

$$q = \frac{[DMPC]}{[DHPC]} \qquad \textit{Eq. 2}$$

DMPC-C_8E_5 samples with theoretical ***q*** - values were prepared from 0.05, 0.10, 0.15, 0.20, 0.30, 0.35, 0.40, 0.45, and 0.50 with a total lipid-detergent concentration kept constant at 5% (w/w). Samples were prepared on a 12 mL volume scale. To ten 15-mL conical tubes the following quantities of DMPC was added to realize the theoretical bicelle ***q*** - values described above. Next, 11.10 mL of Millipore water was added followed by 300 µL of 400 mM HEPE pH 7.4, 4.0 M NaCl buffer. The DMPC mixture was vortexed for several seconds to create a milky, white suspension. To each of the ten samples C_8E_5 was added in quantities that yielded resulting ***q*** - values of 0.05, 0.10, 0.15, 0.20, 0.30, 0.35, 0.40, 0.45, and 0.50, respectively. The samples were vortexed after adding C_8E_5 and turned clear.

To determine the size dependence of DMPC-C_8E_5 lipid aggregates on the total lipid-detergent concentration six samples were prepared on a 12 mL volume scale with a total lipid

concentration of 1, 5, 10, 15, 20 and 25% (w/w). The theoretical ***q*** - value was held constant at 0.25 for all six samples: To six 15 mL conical tubes DMPC was added to produce a final lipid-detergent concentration of 1, 5, 10, 15, 20, 25% (w/w) followed by the appropriate quantity of Millipore water. To each of the six samples, 300 µL of 40X HEPES pH 7.4, NaCl buffer was added to a final concentration of 10 mM HEPES pH 7.4, 100 mM NaCl was also added to each sample. The samples were vortexed to a milky, white suspension and C_8E_5 was added in the proper amounts for a final lipid-detergent concentration of 1, 5, 10, 15, 20, and 25% (w/w) and a theoretical ***q*** - value of 0.25. The samples were vortexed briefly until clear.

Viscosity measurements were carried out on a Cannon Fenske viscometer at a temperature of 20 °C and dynamic light scattering experiments were carried out on an ALV-CGS3 compact goniometer equipped with a 22 mW HeNe laser at a wavelength of 632.8 nm. The scattered light intensity was measured at a detector angle of 90 degrees for eight samples. Diffusion constants for the lipid-detergent aggregates were acquired from the decay of the autocorrelation function, and the hydrodynamic radii were determined by the Stokes-Einstein equation for a spherical particle [25].

2.6 Curve fitting

The shape of the lipid-detergent aggregates was deduced following the detailed method of Mazer and co-workers to characterize mixed micelle formation in bile salt-lecithin solutions. This method was used by Glover and co-workers to deduce the discoidal shape of DMPC-DHPC bicelles [3,23]. The lipid-detergent solutions were treated as monodisperse and non-interacting where the mean scattering intensity, I, is given by the following equation:

$$I = \bar{C}MP \qquad \textit{Eq. 3}$$

where C is the concentration of the lipid-detergent *(w/w)*, M is the molecular weight of the lipid-detergent aggregate, and P describes the scattering form factor. The quantity I/C is proportional to MP. Using this approach, the product MP will have different values depending on the shape of

the lipid detergent aggregate. It follows that the quantity I/C, which is equivalent to MP, can be measured experimentally for DMPC-C_8E_5 aggregates and the shape deduced from a semi-log plot of the normalized I/C versus R_h. The dependence of I/C on R_h was fitted to one of three curves generated for aggregates with a spherical shape (Figure 7 - solid line), disk shape (Figure 7 - dashed line), and a rod shape (Figure 7 - dotted line).

3. Results and Discussion

DMPC-C_8E_5 mixtures were prepared and characterized using analytical techniques each of which would allow us to determine if these mixtures were forming discoidal-type structures similar to the well-characterized DMPC-DHPC bicelles. The latter have been used in solution NMR studies of membrane protein structure and offer the advantage of providing a more native-like lipid bilayer environment [3]. Because membrane protein structure is highly influenced by the surrounding lipid environment, it is important to consider the choice of detergent or lipid system. Other mixed micellar systems have also been adapted successfully for studies of membrane proteins. For example, the human adenosine A_{2A} receptor, a G-protein coupled receptor, has been expressed and isolated in a ligand active form using a mixed micelle system consisting of DDM, CHAPS, and CHS (a stable cholesterol derivative) [17,19]. When choosing a membrane-like mimic to study membrane protein structure and interactions (association or oligomerizaion) it is often necessary to adapt detergents and lipids to the specific protein of interest and a stream-lined predictive method has not yet been successfully established. Initially, we sought to find an alternate system to DMPC-DHPC bicelles that would be more conducive to studying membrane protein interactions using sedimentation equilibrium (AUC). C_8E_5 was chosen as an alternative to DHPC because of its close physical density to that of water, which theoretically requires a lower addition of a density modifier for matching conditions to be achieved. DMPC-DHPC bicelles were density matched using three biocompatible density modifiers: D_2O, glycerol, and sucrose. The goal was to find the concentration and or density at which, using the Lamm equation (Eq. 1), M_{eff}

would be equal to zero. At this point, the respective solutions have been effectively density matched. Using 71.7% (v/v) D_2O, 23.5% (v/v) glycerol, and 0.418 M sucrose bicelles were density matched, but significantly higher levels of each modifier were required (Figure 3). The risk of using modifiers at exceedingly high concentrations and compositions is the risk of generating a concentration gradient in the solution column during sedimentation equilibrium analysis of membrane proteins, which can distort the results. Glycerol and sucrose are particularly susceptible to gradient formation and for that reason we chose to proceed using D_2O.[26] In comparison, DMPC-C_8E_5 aggregates only require 18.4% (v/v) D_2O, offering a significant advantage over using bicelles in this case (Figure 2).

Next, we used ^{31}P-NMR to evaluate the chemical environment of the phosphate group on DMPC. Using this approach we reasoned that if the chemical environment of the phosphate group on DMPC is vastly different in the presence of DHPC versus C_8E_5, then there must be a significant alteration in the arrangement of the lipids between the two systems. To distinguish between the two lipids in the DMPC-DHPC bicelle sample the lanthanide shift reagent, praseodymium (III) chloride was added, which preferentially interacts with longer chain lipids thereby allowing the two phosphorous signals to be differentiated from one another (Figure 4). A higher overall lipid composition was used, 25% (w/w), to generate a detectable signal. An overlay of the two spectra and analysis of their chemical shifts indicate a very slight difference in the chemical environments, but not enough to suggest that the structures of DMPC in either mixture are much different. Light scattering experiments, on the other hand, indicate that their overall shape may actually differ from one another substantially.

In our light scattering analysis samples were prepared at a ***q*** - value of 0.25 at various % total lipid-detergent compositions ranging from 1% up to 25% (w/w). At a total lipid-detergent concentration of 25% (w/w), dynamic light scattering data could not be acquired because the lower limit of detection had been reached and it was not possible to measure the hydrodynamic radius. Interestingly, as the overall concentration of lipid-detergent increased, the hydrodynamic

radius of the aggregates decreased rapidly at first and eventually slowing to a final value of approximately 1 nm (Figure 5, Table 1). From this data it suggests that at higher lipid-detergent concentrations, in spite of a constant ***q*** ratio, the aggregate size decreases. One plausible explanation for this observation is that if we assume that C_8E_5 is in equilibrium with the DMPC-C_8E_5 aggregate, the concentration of available C_8E_5 that is in equilibrium with the lipid-detergent aggregate increases as the total lipid-detergent concentration goes up. When this happens, the resulting size of the aggregates decrease. Compared to DMPC-DHPC bicelles the solution viscosity of DMPC-C_8E_5 mixtures also increased substantially as the lipid concentration increased (Table 1).

From the Stoke's-Einstein equation and the decay of the autocorrelation function, the hydrodynamic radius, R_h, of the DMPC-C_8E_5 aggregates was determined for each lipid-detergent sample and plotted versus the calculated theoretical ***q*** - value. In Figure 6 we observe a substantial increase in the hydrodynamic radius of the lipid aggregate size corresponding to an increase in ***q*** when the total lipid concentration is held constant at 5% (w/w). A similar increase in the hydrodynamic radius was observed previously with DMPC-DHPC bicelles, which initially supported our hypothesis that DMPC-C_8E_5 lipid-detergent structures assume a bicellar-like arrangement [3]. To further investigate the shape of these structures we followed the method of Mazer and co-workers in detail [23]. Using this approach, we generated three theoretical curves that describe the behavior of spherical aggregates (solid line), discoidal aggregates (dashed line), and rod-like aggregates (dotted line). The theoretical molecular weight, M, was calculated for each type of aggregate along with a unique form factor, P. The product of the molecular weight and the form factor, MP, is equivalent to the experimentally determined scattering intensity, I, divided by the lipid-detergent concentration, C, for the DMPC-C_8E_5 structures:

$$\frac{I}{C} = MP \qquad \textit{Eq. 4}$$

For the theoretical curves, the product of the molecular weight and form factor, MP, were calculated and plotted on a semi-log plot versus the hydrodynamic radius for theoretical spheres, disks, and rods. The range for the hydrodynamic radius was determined based on the measured hydrodynamic radius, R_h, for the DMPC-C_8E_5 samples. These R_h values were substituted into the values of R_h for each of the theoretical models and the calculated scattering intensity was plotted in Figure 7. We chose to use dimensions of DMPC that corresponded to the previous findings by Glover and co-workers, which agreed with the theoretical calculations introduced by Vold and Prosser [3,4,27]. For the disk and rod-shaped models, the thickness, t, and the diameter, d, were kept constant at 5 nm, governed by the length of DMPC [3]. In these two models the radius of the disk, r, and the length, L, of the rod-shaped aggregates were adjusted to the measured values of the radius for the DMPC-C_8E_5 aggregates. For the spherical model, the radius, a, was left to float between values of 1 and 10. The data were normalized to the smallest value and ten points were obtained for each theoretical curve. A semi-log plot of MP versus R_h shows that the scattering intensity of the different lipid aggregates decreases going from a sphere to a rod in Figure 7.

For DMPC-C_8E_5 the scattering intensity, I, was divided by the total lipid-detergent concentration, C, for each theoretical $\boldsymbol{q}$ - value. The data were normalized to the lowest scattering intensity and plotted against the measured hydrodynamic radius, R_h. Our analysis revealed that when the normalized scattered light intensity was plotted on a semi-log plot versus the hydrodynamic radius for DMPC-C_8E_5, the resulting curve overlays remarkably well with the curve for a spherical micellar aggregate. This result is consistent with a mixed micellar model and is somewhat surprising considering that DMPC-DHPC lipid aggregates form disk-shaped structures. The DMPC-C_8E_5 aggregate structures do not contain a distinct planar region as in the case of traditional DMPC-DHPC bicelles, which may be a direct result of the presence of the oxygen atoms in the hydrocarbon chain of C_8E_5. The presence of oxygen in the hydrocarbon chain likely

influences the ability of the detergent to assume the physical requirements necessary to create a rim around DMPC in a disk-like arrangement. Specifically, C_8E_5 may not have the ability to pack together as tightly due to repulsive effects of neighboring oxygen atoms in the hydrocarbon chain (Figure 1A).

4. Conclusions

In this study DMPC-C_8E_5 lipid-detergent aggregates/mixtures were prepared at various molar ratios and total lipid compositions. These aggregates were evaluated using different bioanalytical techniques and the measured properties compared to the well-characterized DMPC-DHPC bicellar system. This work was conceived as a result of the need for alternative bicelle systems, which could be used as bilayered cell membrane-like mimics for characterizing membrane protein interactions in a native-like environment. In this study we conclude that DMPC-C_8E_5 lipid-detergent aggregates were better suited to density matching studies in the analytical ultracentrifuge because they require less of the density modifier, D_2O, compared to DMPC-DHPC bicelles. This lipid-detergent system can be especially advantageous when evaluating small membrane proteins, which can be more challenging to study given their low density relative to water and requiring greater centrifugal speeds in sedimentation equilibrium analyses. Interestingly, these structures are predicted to form spherical aggregates rather than discoidal-shaped aggregates like their DMPC-DHPC counterparts. This is the first reported instance where this type of mixture has been prepared and characterized. These mixtures are unique to themselves and warrant further study to better understand their behavior at different temperatures and under different physical conditions. Both lipids are relatively benign, *i.e* biocompatible, and may prove to have desirable properties that may make them applicable to fields like drug delivery. We can also conclude from these studies that there are unique conditions and properties that lipids and detergents molecules must have for their spontaneous assembly into bicelles and not all detergents are suited to adopt this geometric conformation. A separate analysis is required to

ascertain the precise properties of lipids that can enable a better prediction of the requirements for discoidal bicellar assembly.

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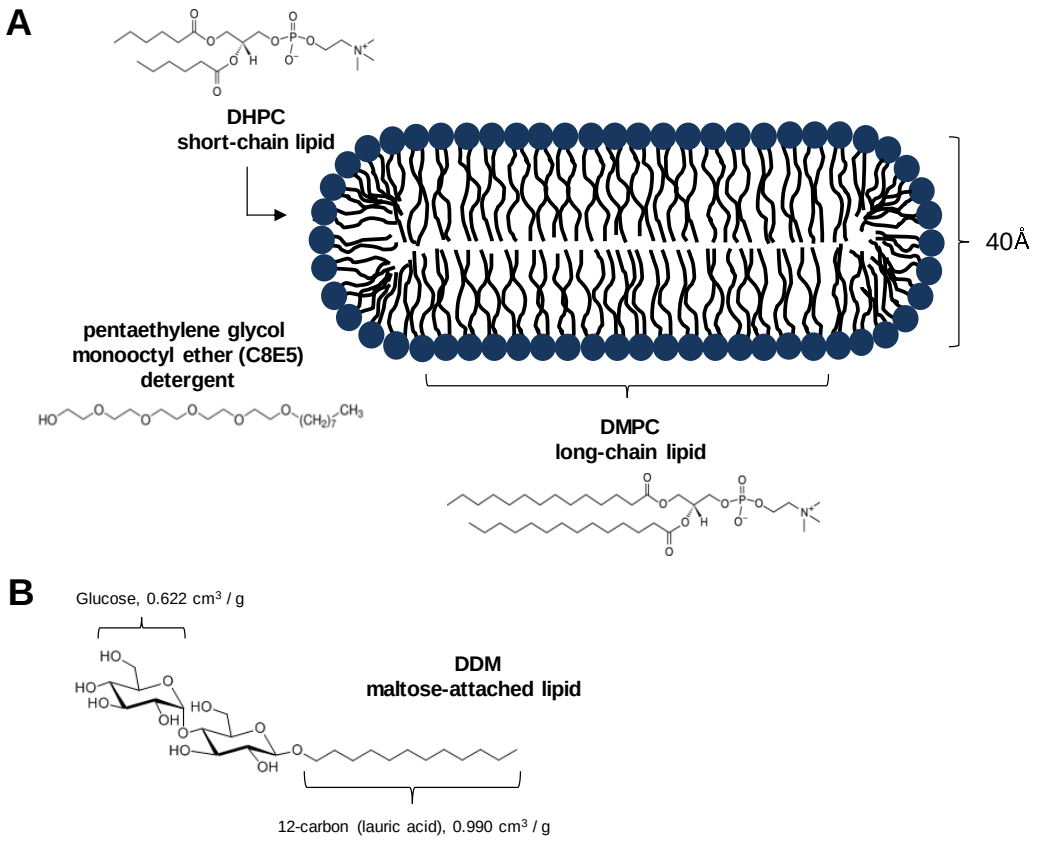

Figure 1. A) Schematic diagram of a bicelle containing DMPC and DHPC. Samples were prepared replacing DHPC with C_8E_5 predicted to replace DHPC at the rim of bicelle. B) Dodecyl-β -D-maltoside (DDM) lipid with dense maltose sugar headgroup; glucose = 0.622 cm^3 / g, 12-carbon chain = 0.990 cm^3 / g. DDM has been used in mixed micelle systems to study the human adenosine A_{2A} receptor expressed and isolated from *S. cerevisiae*.

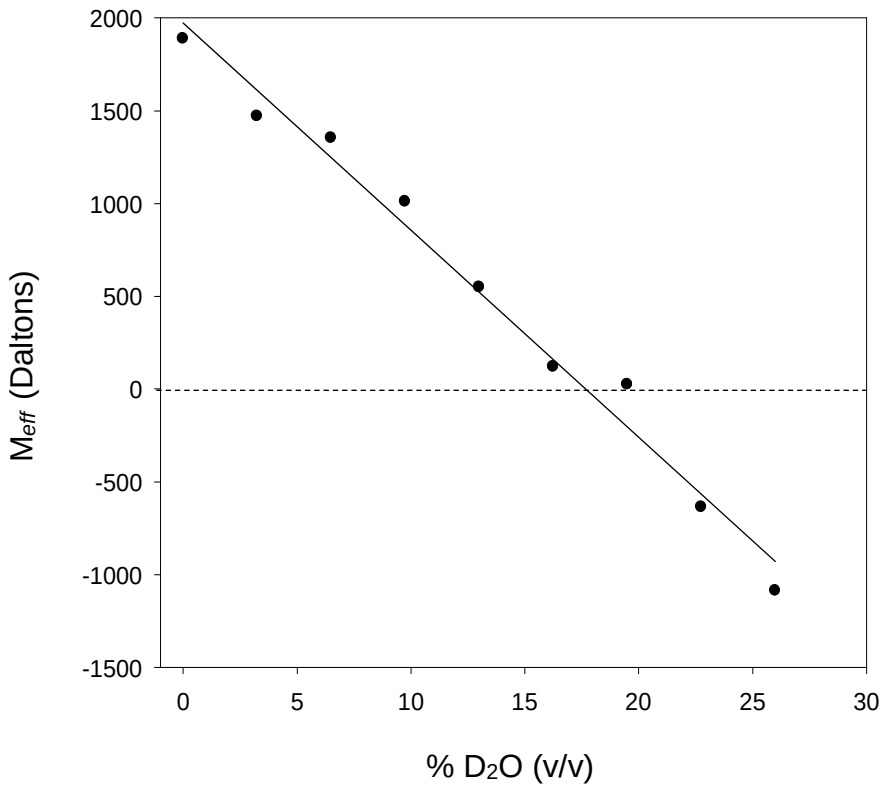

Figure 2. M_{eff} versus D_2O concentration. Approximately 18.4% D_2O is required to density match DMPC-C_8E_5 lipid-detergent aggregates.

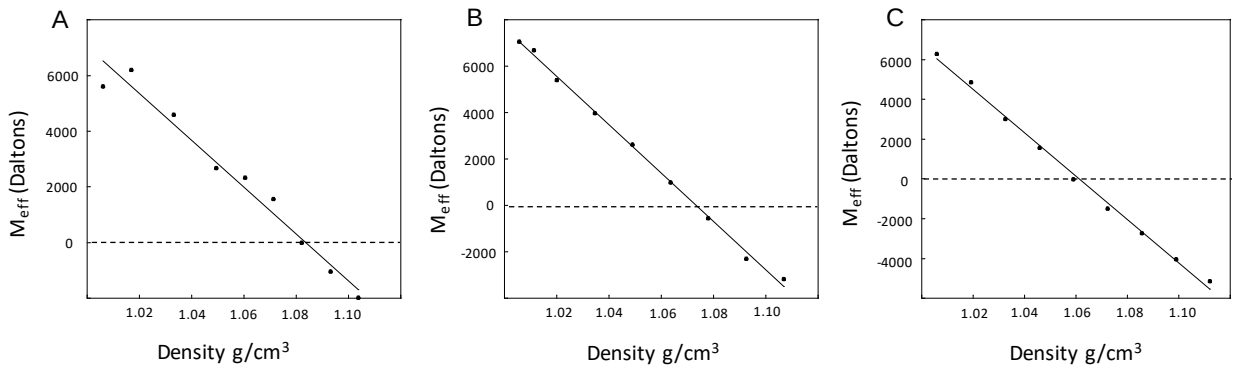

Figure 3. Density matching using three biocompatible density modifiers: A) D_2O B) glycerol C) sucrose. Approximately 71.7% (v/v) D_2O, 23.5 % (v/v) glycerol and 0.418 M sucrose is required to density match DMPC-DHPC bicelles.

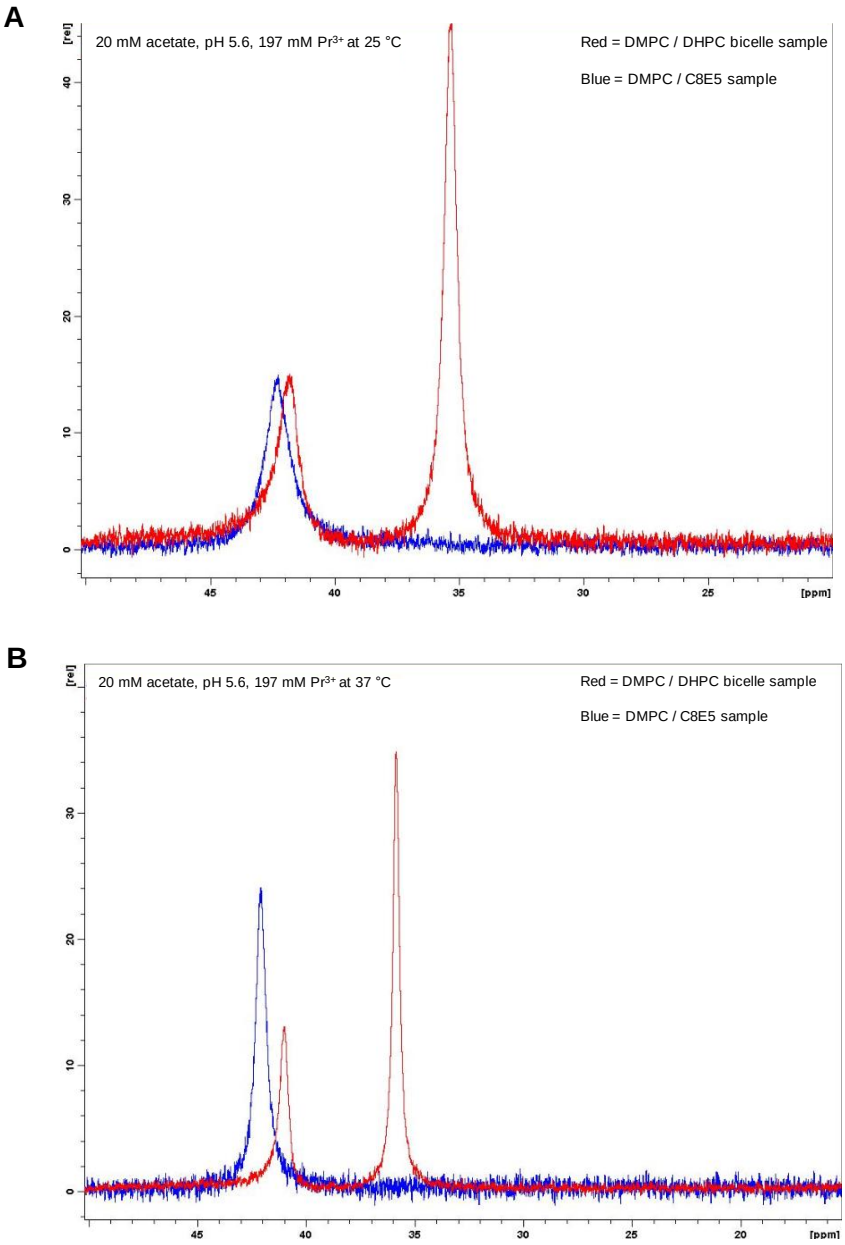

Figure 4. ^{31}P-phosphorus NMR spectra of C_8E_5 (blue) and DMPC (red). The chemical shift of DHPC is 35.1 ppm and the chemical shift of DMPC appears at 42.0 ppm for DMPC in DMPC / DHPC bicelles and 42.5 ppm in DMPC / C_8E_5 bicelles. Panel A is the sample analyzed at 25 ºC and Panel B is the sample analyzed at 37 ºC. *Note: Data was also collected at 31 ºC showing chemical shifts similar to 37 ºC.* (*spectrum not shown)*.

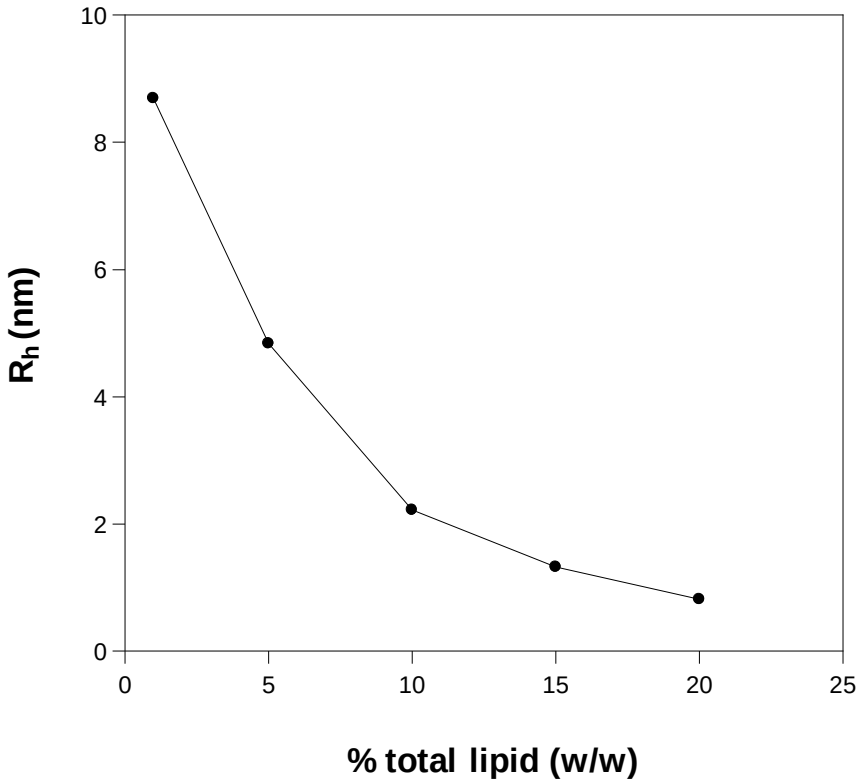

Figure 5. R_h versus % total lipid (w/w). Particle size decreases as total lipid concentration increases.

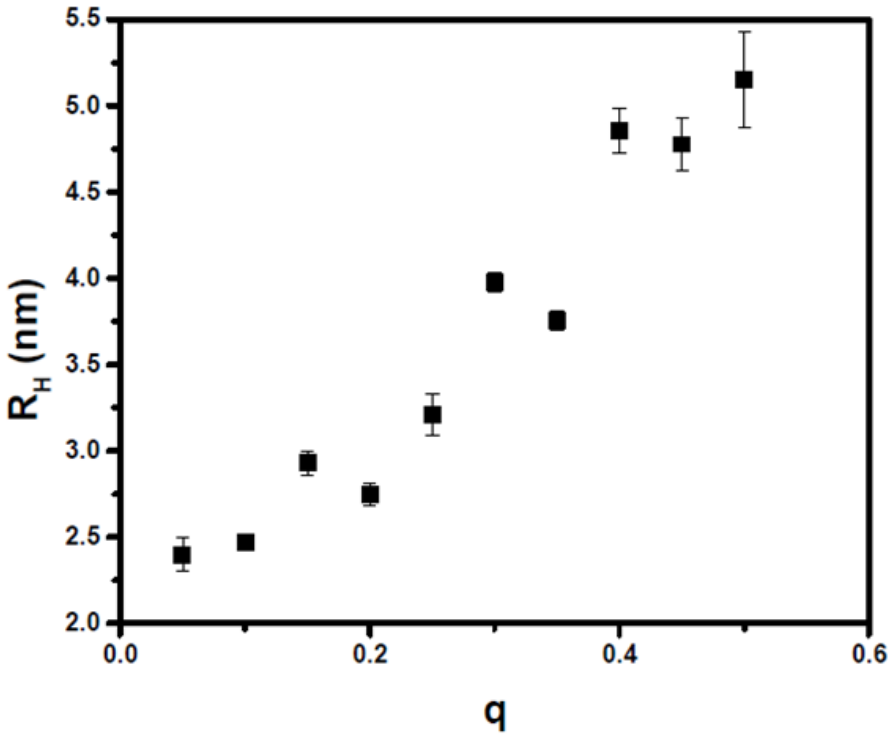

Figure 6. R_h versus theoretical ***q***. Increasing theoretical ***q*** corresponds to an increase in the hydrodynamic radius of the particle.

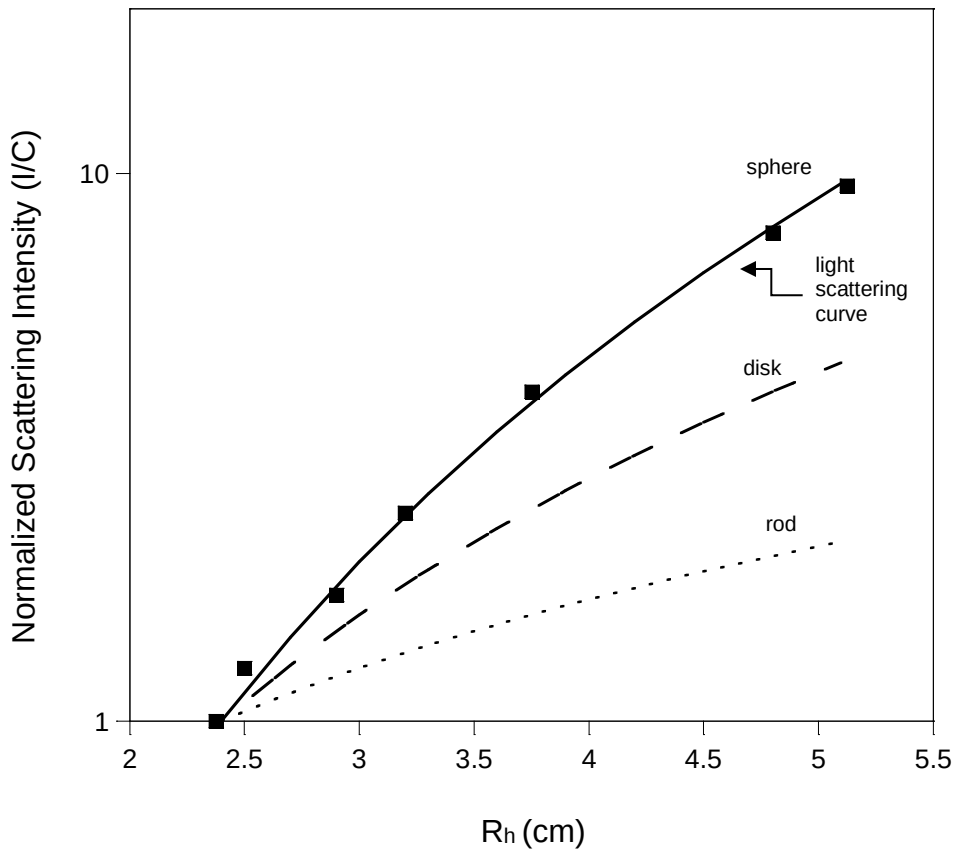

Figure 7. Semi-log plot of normalized scattering intensity versus radius of hydration, R_h. The solid line represents the model of a spherical particle, the dashed line represents the model of a disk, the dotted line represents the model of a rod. The dotted-square line represents the DLS data. The DLS data fit the curve for the sphere indicating the shape of the DMPC / C_8E_5 aggregates are spherical.

Table I. Measurements of viscosity and hydrodynamic radius in relation to increasing lipid concentration

q = 0.25, % lipid (w/w)	***Dynamic Viscosity (Pa*s)***	***Viscosity (cP)***	***R_h (nm)***
1	0.00109	1.09144	8.69
5	0.00129	1.28765	4.84
10	0.00182	1.82314	2.22
15	0.00286	2.85859	1.33
20	0.00441	4.41406	0.82
25	0.00674	6.73514	N/A