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Dependence of liquid crystal morphology on phospholipid hydrocarbon length

H. Rutherford [a], N.H. Low [a], F. Borondics [b], T. Pedersen [b], M.A. Rogers [c,*]

[a] Department of Food and Bioproduct Science, University of Saskatchewan, 51 Campus Dr. Saskatoon, SK, S7N5A8, Canada
[b] Canadian Light Source, Saskatoon, SK, Canada
[c] Department of Food Science, Rutgers University; The State University of New Jersey, New Brunswick, NJ, Canada

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ABSTRACT

The liquid crystal morphologies of symmetrical diacy phosphatidylcholine liposomes examined in this research study were found to be dependent on saturated hydrocarbon chain length. Both powder X-ray diffraction and synchrotron mid-IR spectromicroscopy indicate that phosphatidylcholines with short hydrocarbon tails (i.e. ten and twelve carbons) are more likely to form unilamellar liposomes while those with long hydrocarbon tails (i.e. eighteen and twenty carbons) are more likely to form multilamellar liposomes. Hydrocarbon chain lengths of fourteen and sixteen represent a transitional zone between these two liquid crystal morphologies. The FTIR spectra where a shoulder develops on the peak at wavenumber 1750 cm^{-1} particularly highlights the change in the packing of adjacent molecules in the transitional zone.

1. Introduction

Amphiphilic molecules have the ability to associate into numerous structures in aqueous solutions depending on molecular packing considerations. The ability to pack into supramolecular structures is governed by the hydrophobic attraction at the hydrocarbon–water interface. As the molecules associate, the hydrocarbon chains pack together while the polar entity of the molecule remains in contact with the aqueous phase. Hydrophilic, ionic or stearic repulsion of the polar head groups can further alter the packing of the molecules [1].

These contrasting parameters are a result of the amphiphilic nature of the molecules and dictate the supramolecular structure [2]. The favoured structure of amphiphilic molecules is determined by: the optimal effective area of the head group (a); the volume of the hydrocarbon chain(s) (v), which is/are fluid and incompressible; and the maximum effective length of the chain (l_c) [1]. Depending on these packing considerations, a variety of supramolecular structures are possible [1]. Liposomes are spherical supramolecular structures of phospholipids composed of at least one phospholipid bilayer [1].

Israelachvili has illustrated that in order for amphiphilic molecules to assemble into spherical vesicles, the surface area (a_0) must be sufficiently large, while the hydrocarbon volume (v) must be sufficiently small and the radius of the supramolecular structure (R) must not exceed the critical length of the amphiphilic molecule [1]. Based on geometric considerations, for a sphere of radius, R, the mean aggregation number is equal to:

$$M = \frac{4\pi R^2}{a_0} = \frac{4\pi R^3}{3v} \quad (1)$$

this may be rearranged to:

$$R = \frac{3v}{a_0} \quad (2)$$

hence, the geometric constraint for a spherical vesicle may be calculated by:

$$\frac{v}{a_0 l_c} \quad (3)$$

where $R = l_c$ Israelachvili has determined mean dynamic packing parameters for different liquid crystal morphologies [1]. Amphiphilic lipids with two hydrocarbon chains are expected to form bilayers and liposomes due to their large hydrocarbon volume resulting in a $v/a_0 l_c$ in the range of 1/2 to 1 [1].

From the earliest investigations of phospholipid liquid crystal structures, particularly liposomes, multiple bilayers structures have been observed [3,4]. Research in the area of liposomes quickly began to focus on the ability of these structures to effectively encapsulate compounds for a variety of applications including drug delivery [3–5]. Undoubtedly, the most efficient way to study liposomes for encapsulation purposes is to prepare homogeneous suspensions of unilamellar liposomes, often this is accomplished using sonication, membrane extrusion or microfluidization [5–7]. Particularly when conducting research on liposomes for drug delivery applications, control of both size and structure is of great

* Corresponding author.
E-mail address: rogers@aesop.rutgers.edu (M.A. Rogers).

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concern since these parameters will affect the performance of the drug delivery system *in vivo* [5,8].

Hydrocarbon chain length has been shown to have a positive linear relationship with bilayer thickness [9]. The influence of hydrocarbon length on liposome size can be overcome with processing; liposomes of a minimal size can be formed from phospholipids with differing hydrocarbon chain lengths [10]. The affect of the hydrocarbon chain length has not only been shown to influence the morphology of the liquid crystalline structure but also the fluidity of the bilayer [1]. While the formula for geometric packing parameter (Eq. (3)) can be used to predict supramolecular structures, it does not allow for the prediction of the structure of multilamellar liposomes. While unilamellar liposomes have been considered to have more utility than their multilamellar counterparts, there is great value in investigating the preferential aggregation of phospholipids with differing hydrocarbon chain lengths into supramolecular structures without the aid of external factors such as homogenization, filtration or ultrasonics.

2. Materials and methods

The following synthetic symmetrical diacyl phosphatidylcholines were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and used as received: 1,2-didecanoyl-*sn*-glycero-3-phosphocholine (PC10), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (PC12), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (PC14), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC16), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (PC18), and 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine (PC20). Liposomes were formed using a dry film gentle hydration method [11,12]. Samples were prepared in triplicate. The film was prepared by dissolving 0.1 mM of phosphatidylcholine in 2 mL of chloroform (Sigma–Aldrich, St. Louis, MO, USA) in a 50 mL round bottom flask. A Buchi rotary evaporator (Flawil, Switzerland) with the water bath at 55 °C was used to evaporate the chloroform under reduced pressure leaving a thin dry film of phosphtidylcholine. Two milliliters of HEPES buffered saline (20 mM HEPES, 150 mM NaCl) (Sigma–Aldrich, St. Louis, MO, USA) was added to the film and was mixed using the rotary action of the Buchi rotary evaporator with the water bath temperature set above the transition temperature of the particular phosphatidylcholine being used. The resulting liposome suspensions were sonicated using a Branson 2510 bath sonicator (Danbury, CT, USA) at room temperature for 10 min at 40 kHz (i.e. until no large aggregates were visible in the suspension).

A Nikon Eclipse E400 light microscope (Nikon Instruments Inc., Melville, NY, USA) equipped with a Nikon DS-Fi1 color camera (Nikon Instruments Inc., Melville, NY, USA) and a 40× lens and condenser (Nikon Instruments Inc., Melville, NY, USA) were used for light microscopy. Images were captured at a resolution of 2560 × 1920 pixels using a Nikon DS-FiL color camera. Images were analyzed using Adobe Photoshop Extended CS4.0 (Adobe Systems Inc., San Jose, CA, USA).

Light scattering measurements (hydrodynamic size and surface area) were performed using a Malvern Hydro 2000S Mastersizer (Malvern, Worcestershire, UK). Rheological experiments were performed with a Thermal Analysis AR-G2 rheometer (New Castle, DE, USA) using a 2° 40 mm acrylic cone geometry. Frequency sweep measurements were conducted at a constant oscillatory stress of 1 Pa and a range of frequencies from 1 to 100 rad/s. Continuous ramp experiments were performed with an increase in shear rate (1/*s*) from 0.01 to 1000.

Fourier transform infrared (FTIR) spectra were collected at the Canadian Light Source (Saskatoon, SK, Canada) at the mid-IR spectromicroscopy beamline (01B1-1). The end station was comprised of a Bruker Optics IFS66 v/S interferometer coupled to a Hyperion 2000 IR microscope (Bruker Optics, Billerica, MA, USA). A drop of sample was placed between two CaF_2 optical windows (25 mm diameter, 2 mm thick). Light is focused on the sample using a 15× magnification Schwarzschild condenser, collected by a 15× magnification Schwarzschild objective with the aperture set to a spot size of 40 mm × 40 mm and detected by a liquid nitrogen cooled narrowband MCT detector utilizing a 100 mm sensing element.

A KBr-supported Ge multilayer beamsplitter was used to measure spectra in the mid-infrared spectral region. Measurements were performed using OPUS 6.5 software (Bruker Optics, Billerica, MA). The measured interferograms were an average of 512 scans and were recorded by scanning the moving mirror at 40 kHz (in relation to the reference HeNe laser wavelength of 632.8 nm). The wavelength range collected was 690–7899 cm^{-1} with a spectral resolution of 4 cm^{-1}. Single channel traces were obtained using the fast Fourier transform algorithm, with no zero filling, after applying a Blackman–Harris 3-Term apodization function.

A Rigaku Multiplex Powder X-ray Diffractometer (Rigaku, Japan) with a 1/2 degree divergence slit, 1/2 degree scatter slit, and a 0.3 mm receiving slit, was set at 40 kV and 44 mA to determine the polymorphic form and long spacings of the phosphatidylcholine vesicles. Scans were performed from 1 to 30 degrees 2θ at 0.2°/min.

Statistical analyses were performed with GraphPad Prism 5 (San Diego, CA, USA).

3. Results and discussion

The affect of sonication time on liposome size was assessed prior to deciding the final preparation method. Ten minutes was sufficient to disrupt large visible aggregates in the suspension, after ten minutes no significant changes in the size of the vesicles were noted. The application of heat during the mechanical agitation of the suspension ensured that all the lipid was removed from the glass surface and suspended in aqueous solution.

Regardless of hydrocarbon chain length, phosphatidylcholines from C10 to C20, were observed to form spherical aggregates (Fig. 1). Visualization, using light microscopy, of these spherical aggregates could not conclusively discern whether the vesicles were composed of single or multiple bilayers. The lower hydrocarbon chain lengths, C10-14, appear to have small spherical vesicles which have aggregated during sample preparation (Fig. 1A–C). Fig. 1D–F, representing phosphatidylcholines with hydrocarbon chain lengths greater than 16 carbons, appeared to have a slightly different morphology. As a note, it is not surprising to see a cylindrical and spherical structures in Fig. 1D as it is possible that both structures can exist in thermodynamic equilibrium [1].

Previous research has revealed a positive linear relationship between hydrocarbon chain length in phospholipids and bilayer thickness [8,9,13]. Assuming that a thicker bilayer would require a larger radius of curvature, it was expected that liposome size would increase with increasing hydrocarbon chain length. Ten randomly selected vesicles on three different micrographs were analyzed using Photoshop; the initial measurement of diameter was taken in pixels and converted to microns using a calibrated scale bar. This number-mean diameter (D[1,0]) was converted to a volume-moment mean diameter (D[3,4]) for comparison with the collected light scattering data. The values of the D[3,4] indicated that below C14 there was no significant effect on vesicle size as a function of hydrocarbon chain length; however there was a drastic change in vesicle size above C16 (Fig. 2). Two separate trends within the vesicle diameter collected using light microscopy were observed: the first being a plateau from PC10 to PC14 and the second being a size increase from PC14 to PC20 including a positive linear trend within the C14–C18 hydrocarbon length range (Fig. 2).

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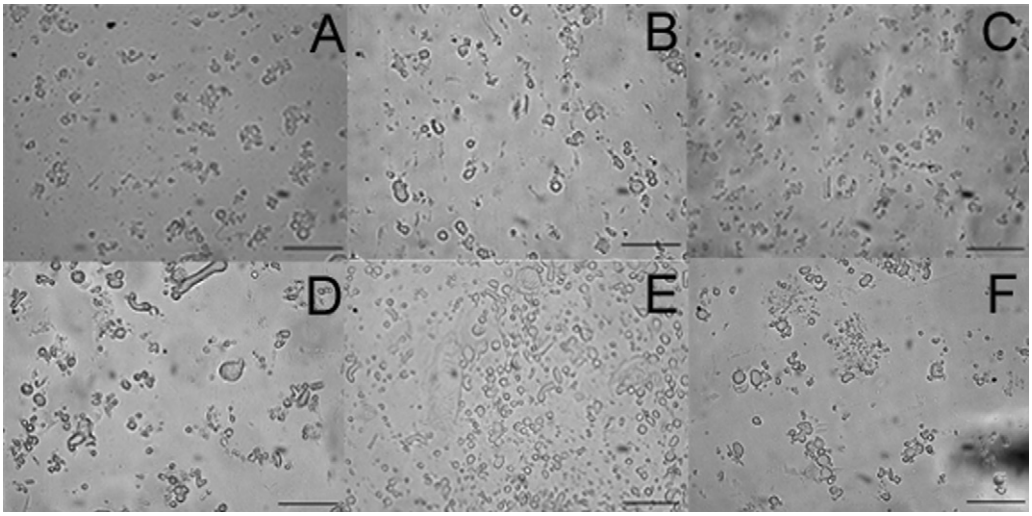

Fig. 1. Light micrographs of vesicle suspensions composed of PC10 (A), PC12 (B), PC14 (C), PC16 (D), PC18 (E), and PC20 (F). Scale bar indicates 5 μm.

To confirm the vesicle size trend, dynamic light scattering was used to determine the size of the vesicles in solution. The raw percent volume versus particle size (Fig. 3) indicated that for short hydrocarbon chains (i.e., less than C14) the distribution of vesicle size was similar (Fig. 3A–C). A bimodal distribution was observed which suggests, in concordance with the light micrographs, that there were small vesicles and aggregates of small vesicles. Above C14 (Fig. 3D–F) the distribution shifts to significantly larger particle sizes.

The calculated *D*[3,4] determined using light scattering (Fig. 4A) showed similar trends to those values determined using light microscopy (Fig. 2). A distinct linear tread in vesicle size was observed above C14. Below a hydrocarbon chain length of C14, no significant effect of chain length on vesicle size was observed. Further, the surface area of the vesicles showed significant differences in the surface area of the vesicle suspensions that do not correspond to this assumption (Fig. 4B). The parabolic relationship of surface area and hydrocarbon chain length indicates that the same type of structure is not being formed by the different chain length phosphatidylcholines. Particularly, the increase in surface area from PC10 to PC12 and the abrupt decrease at PC14 indicates a change in the structural dynamics of the vesicle structure. This suggests that the packing arrangement may be significantly affected by the hydrocarbon chain length resulting in a transition from a unilamellar to multilamellar liposomes.

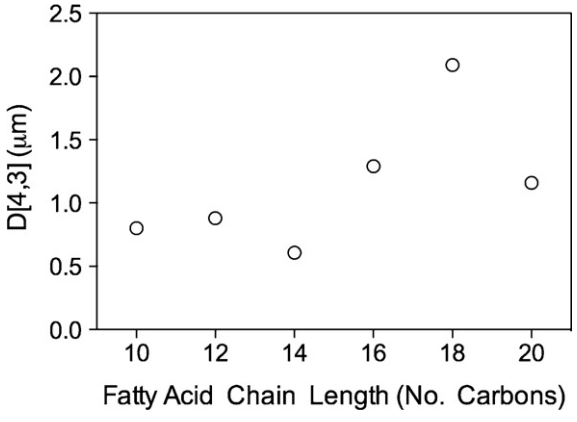

Fig. 2. Volume–moment mean diameters of phosphatidylcholine vesicles calculated using pixel measurements obtained from light micrographs.

X-ray powder diffraction data indicated that the phosopholipid head groups were arranged with a short spacing observed at ~4.2 Å (Fig. 5A and B). However, phospholipids with longer hydrocarbon showed a slightly smaller head group spacing ($d \sim 4.1$ Å) (Fig. 5A) compared to the shorter hydrocarbon side chains ($d \sim 4.3$ Å) (Fig. 5B). This is attributed to the smaller vesicle, which in turn results in a greater surface curvature, resulting in an increase in the short spacing.

X-ray diffraction patterns confirm the transition from unilamellar to multilamellar structure with an increase in hydrocarbon chain length (Figs. 5 and 6). This is evident by the 0 0 1 peak at 65.03 Å in Fig. 5A. This indicates that the liposome has a bilayer which is equivalent to two times the length on a single phospholipid. Further, the presence of the higher order reflections at 33.9 Å (0 0 2), 22.6 Å (0 0 3), and 16.9 Å (0 0 4) and 10.9 Å (0 0 6) indicate a liposome with multiple bilayers (Fig. 5A). As well, there are doublets peaks observed at 22 Å and 16 Å suggesting two species are present. Conversely, a single long spacing at 29.5 Å was observed for the C12 phospholipid suggesting that the liposome consists of a single bilayer of phospholipid. This is confirmed by the long spacing being equal to the length of the phospholipid as well as the lack of higher order reflections (Fig. 5B).

Synchrotron mid-IR spectroscopy reveals a change in the short spacing of the head groups at the low and high end of the hydrocarbon lengths examined. The FTIR data indicates differences in both the head group and hydrocarbon chain packing for saturated phosphatidylcholines (Fig. 6). The peak at ~1750 cm^{-1} represents the C=O stretching of the ester bond where the hydrocarbon chain meets the head group [14]. The appearance of a shoulder on the 1750 cm^{-1} peaks, for PC 14 and PC 16, indicates that the head group is more highly confined (i.e., have a greater amplitude of hydrogen bonding). This indicates a transition in the structure of liposomes formed below a chain length of 14 and above a chain length of 16. PC 14 and PC 16 can be considered to have chain lengths within a transitional zone. This result supports the observation in X-ray diffraction patterns (Fig. 5A) where the phospholipids with long hydrocarbon chains have a smaller headgroup spacing (i.e. pack more closely). Logically, the multilamellar liposomes have a larger radius than the unilamellar liposomes, resulting in less torsion applied to the adjacent molecules. As a result, in a multilamellar liposome configuration adjacent molecules are closer and interact more with each other.

The hydrogen bonding associated with water make it difficult to discern the CH_2 (symmetric stretch ~ 2850 cm^{-1}; antisymmet-

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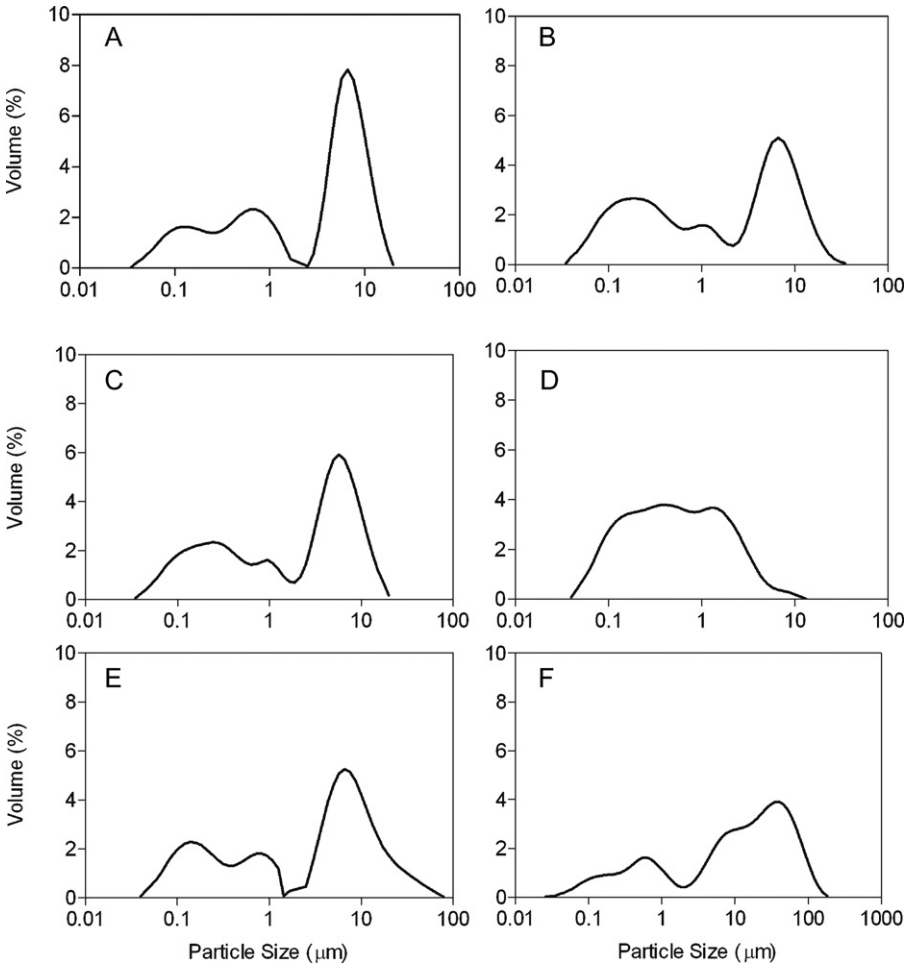

Fig. 3. Light scattering intensities of phosphatidylcholine vesicle suspensions with varying hydrocarbon chain lengths (A) PC10, (B) PC12, (C) PC14, (D) PC16, (E) PC18 and (F) PC20.

ric stretch ~2920 cm^{-1}) and CH_3 (symmetric stretch was not evident; antisymmetric stretch ~2957 cm^{-1}) bands of the hydrocarbon chain therefore deuterated water was used to eliminate the interference of this spectral region (Fig. 6B) [14]. Jiang et al. observed the formation of phospholipid bilayers on a titanium dioxide surface [14]. The system presented here differs, though the differences caused by the curvature of the bilayers in a liposomal system is considered to be negligible. Jiang et al., observed that when PC16 formed a monolayer the CH_2 symmetric stretch was observed at 2851 cm^{-1}, when the phosopholid layer increased to 3 layers the band shifted to 2850 cm^{-1} and at 5 layers it band was observed at 2849 cm^{-1} [14]. Similar trends were observed for the CH_2 antisymmetric stretch where 1 layer had a band at 2919 cm^{-1}, at 3 layers 2919 cm^{-1} and at 5 layers it shifted to 2918 cm^{-1} [14]. Fig. 6B indicates that above C14 there is a shift of both the CH_2 antisymmetric and symmetric stretches to lower wavenumbers corresponding to those observed as the layer thickness went from one phospholipid to multiple phospholipid layers. The shift in the CH_2 bands, along with the powder X-ray diffraction, concur and suggest, in this system, that there is a transition form unilamellar to multilamellar structures above a hydrocarbon chain length of 14 carbons.

While the majority of liposomes in the PC 10 sample are unilamellar, it is likely that within each suspension both unilamellar

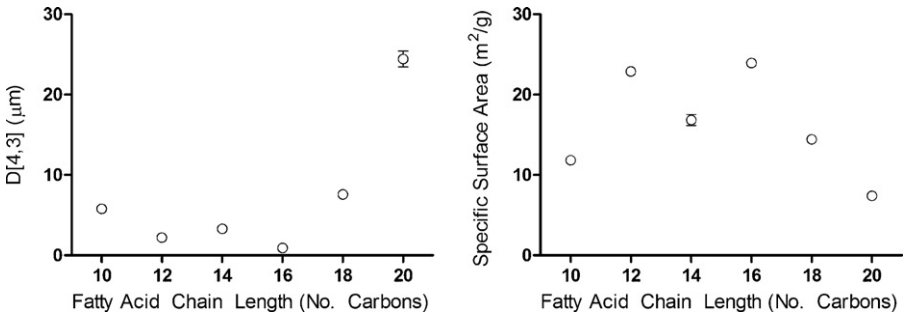

Fig. 4. Volume–moment mean diameters and surface area of phosphatidylcholine vesicles calculated from dynamic light scattering measurements. Error bars equal to one standard deviation.

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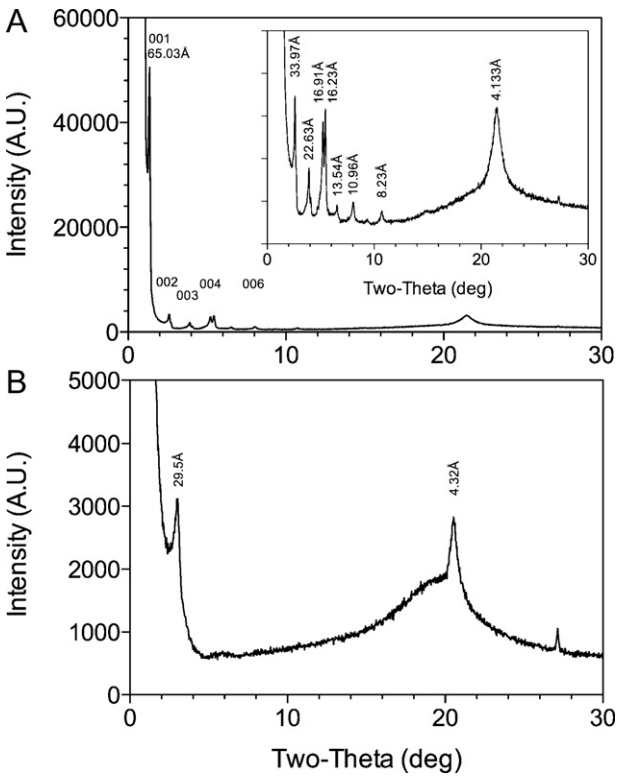

Fig. 5. Powder X-ray diffraction data for PC18 (A) and PC12 (B).

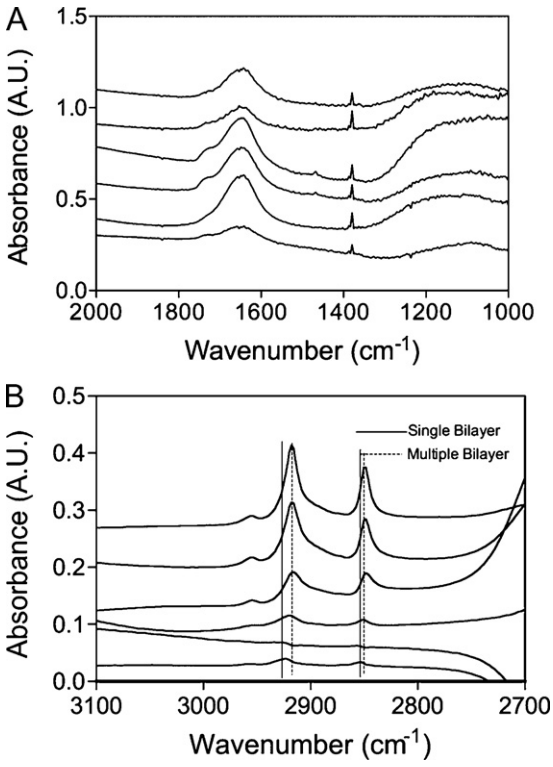

Fig. 6. FTIR spectra for PC 10 (bottom spectrum) to PC 20 (top spectrum) vesicle suspensions in water (A) and deuterated water (B).

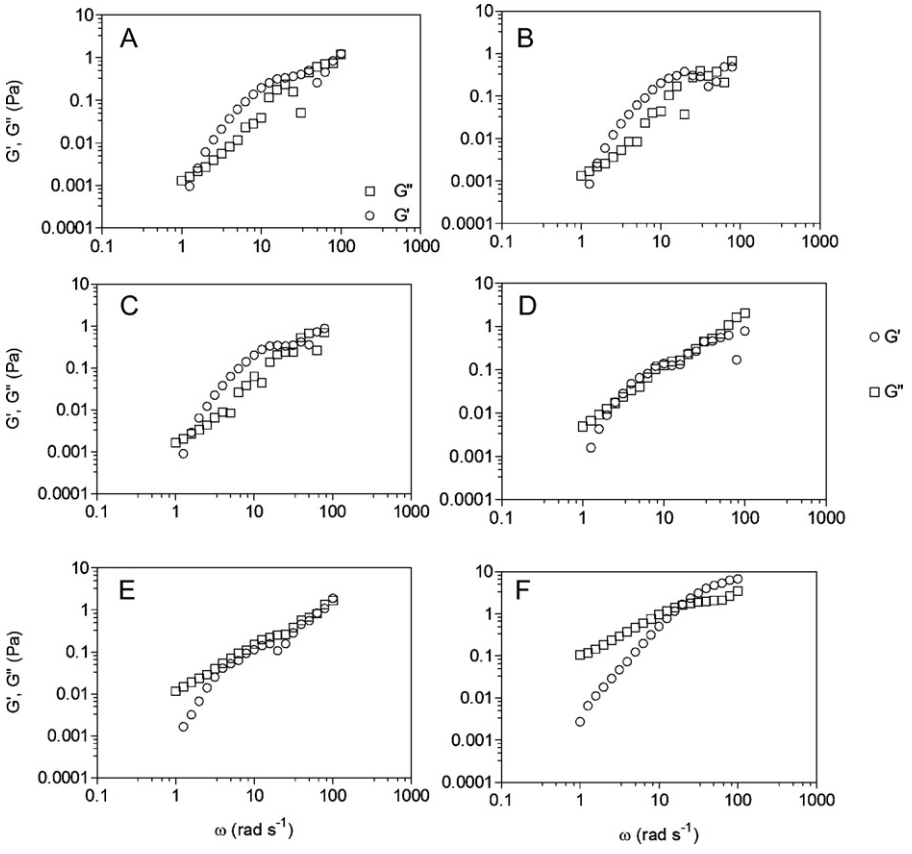

Fig. 7. Oscillatory frequency measurements within the linear visco-elastic region for PC10 (A), PC12 (B), PC14 (C), PC16 (D), PC18 (E) and PC20 (F).

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Table 1
Value of the scaling parameter for the storage ($G' = a\omega^b$) and loss modulus ($G'' = c\omega^d$).

Hydrocarbon length	a (Pa s^{-1})	b (n.u.)	c (Pa s^{-1})	d (n.u.)
10	1.12×10^{-2}	1.17	5.96×10^{-4}	2.02
12	1.33×10^{-2}	1.10	8.10×10^{-4}	1.89
14	1.19×10^{-2}	1.17	3.69×10^{-3}	2.12
16	1.18×10^{-2}	0.98	9.09×10^{-2}	1.08
18	3.60×10^{-3}	1.88	1.074×10^{-1}	0.94
20	4.00×10^{-3}	2.15	1.431×10^{-1}	1.02

and multilamellar liposomes exist. Previous research has shown that multilamellar liposomes are present when samples are prepared using the gentle hydration method. Further investigation would be required to assess the composition of samples based on lamellarity. For the purposes of this study, the trends discussed will be determined using the mean lamellarity indicated by the both the X-ray diffraction data and FTIR.

Oscillatory measurements were used to assess the storage and relaxation modulus of the phospholipid suspensions, storage modulus (G') and loss modulus (G'') respectively (Fig. 7). The behaviour of the liposomal suspensions behave as dilute solutions [15]. The crossover of the G' and G'' values at the middle of the frequency range indicates that the suspensions are behaving more similar to a solid as it is subjected to higher frequencies. PC16, PC18 and PC20 suspensions exhibit this transition. This indicates that multilamellar structures exhibit more solid-like properties under oscillatory stress than the liposomes formed using PC10, PC12 and PC14. By using this simple scaling relationship, a transition occurs as the hydrocarbon length increases. At this point an order of magnitude decrease in the G' scaling constant (a) and an increase in the order of magnitude in G'' (b) is observed (Table 1). This observation confirms that regardless of the phospholipid utilized it has a solution behaviour as a dilute solution. Further, phospholipids with side chains less than C16, form numerous, small diameter liposomes which are capable in establishing an elastic network compared to the few large multilayer liposomes formed with long phospholipid side chains.

4. Conclusions

Phosopholipid supramolecular aggregates may be modified from unilamallar to multilamellar liposomes by altering the carbon number of the hydrocarbon chain. There is a distinct transition from unilamellar to multilamellar liposome where below a chain length of 14 unilamellar liposomes form and above a chain length of 16 multilamellar liposomes form. Evidence from FTIR spectra, particularly, indicates chain lengths of 14 and 16 represent a transitional zone in the liquid crystal morphology of phospholipid liposomes. Understanding and characterizing this structural transition may have utility in investigations of the aggregation of amphiphilic molecules.

Acknowledgments

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