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Analysis of Trisiloxane Phosphocholine Bilayers

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Abstract

We have synthesized unique siloxane phosphocholines and characterized their aggregates in aqueous solution. The siloxane phosphocholines form nearly monodisperse vesicles in aqueous solution without the need for secondary extrusion processes. The area/lipid, lipid volume and bilayer thickness were determined from small angle X-ray scattering experiments. The impetus for the spontaneous formation of unilamellar vesicles by these compounds is discussed.

Keywords

siloxane phosphocholine; unilamellar vesicles; small-angle x-ray scattering; dynamic light scattering

Introduction

Phospholipids are involved in the regulation of cellular functions, they behave as second messengers, and as substrates for phospholipases, lipid kinases, and phosphatases.⁽¹⁾ As a result they are a major component of cellular membranes.

Phospholipid molecules possess three distinct regions that are amenable to chemical modification and can be used to generate interesting lipid structures. The hydrophilic phosphate head group can be linked to one of several moieties such as choline, ethanolamine, glycerol, inositol, or serine. Modification of the head group, in the form of PEGylation for example, can be performed to tune the hydrophilicity of the lipid head group,

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discourage enzymatic hydrolysis, and allow for extended circulation times of lipid nanoparticles *in vivo*.(2) Modifications to the fatty acid (FA) chains are commonly introduced to incorporate fluorophores enabling phospholipase kinetic experiments to be performed.(3) The incorporation of short fluorinated segments of FA tails has conferred higher stability and longer circulation times *in vivo*. (4),(5)

Phospholipids with long chain fatty acid tails (> 10 carbons) self-assemble into multilamellar vesicles (MLVs) in aqueous solutions. Unilamellar vesicles (ULVs) differ from MLVs in that circulation times can be extended, making ULVs better suited as delivery vehicles. From a delivery perspective small ULVs, ranging in size from 50-150 nm and with low size polydispersity, are desirable. Current methods for preparing ULVs include sonication or tedious extrusion procedures, which can be time consuming and require expensive specialized equipment.(6) Spontaneous vesicle formation has been observed previously and vesicles formed in this manner offer great potential for drug delivery.(5)–(9)

In addition to formation of vesicles from phospholipid systems, there are a number of reports of similar structures being formed from siloxane surfactants.(10)–(17)

We were interested in synthesizing silicon-containing biomolecules and evaluating their spontaneous self-assembly behaviour. The hydrophobic nature of silicones would seem to compliment the hydrophobic fatty acid tails of lipids, and in particular phospholipids. To date, however, the only phospholipids containing silicon that have been reported contain a single chain terminated with a chlorodimethylsiloxyl group to allow for covalent modification of solid substrates (**1**, Figure 1).(10)

In this paper we report the biophysical properties of two siloxane-containing phosphocholines (SiPCs, Figure 1).(19)(20)

Experimental Section

SiPC aqueous dispersions

SiPCs were prepared as previously described.(19),(20) Aqueous dispersions of SiPC were prepared by five freeze-thaw cycles to give opaque suspensions of vesicles suitable for SAXS analysis. Dispersion of particle size was determined by dynamic light scattering (DLS) with a Nano Zetasizer ZS90 (Malvern Instruments, Worcestershire, U.K.).

Preparation of ULVs

1-Palmitoyl-2-oleoyl-*sn*-3-glycercophosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-3-glycercophosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. POPC and 5 mol% POPG were co-dissolved in a chloroform:methanol (3:1) mixture to minimize pauci-lamellar vesicle contamination. Solvent was removed under a gentle stream of dry nitrogen gas then was put under vacuum for 8 hours. POPC:POPG films were hydrated with ultrapure water to a concentration of ~30 mg/mL. ULVs were prepared using a manual miniextruder (Avanti Polar Lipids, Alabaster, AL), assembled with a 100 nm pore-diameter polycarbonate filter. The lipid suspension was passed through the filter 31 times. SAXS data were collected at the P12 BioSAXS beamline at the storage ring

PETRA III (synchrotron DESY, Hamburg)(23) at a temperature of 20°C. SAXS data were visualized, averaged and the background was subtract using ATSAS(21) and modelled using the method of Pabst *et al.*(22) The data was fit utilizing a standard non-linear least-squares fitting (NLSF) scheme within Origin (OriginLab, USA). Vesicle size was determined by dynamic light scattering (DLS) with a Nano Zetasizer ZS90 (Malvern Instruments, Worcestershire, U.K.).

Transmission Electron Microscopy (TEM)

Images were acquired using a FEI T12, 120 kV, LaB6-cathode TEM (Hillsboro, Oregon). Samples were spotted on the TEM grids at 22°C and 99% relative humidity using a Leica EM GP plunge freezer (Wetzlar, Germany).

Results and Discussion

Liposomal preparations of SiPCs **2** and **3** were prepared using a freeze-thaw procedure, skipping extrusion through a pre-sized membrane, in order to characterize their spontaneous assembly in aqueous media. Liposomes were characterized by dynamic light scattering (DLS) and small angle X-ray scattering (SAXS) and compared to the prototypical phospholipid, POPC doped with 5 mol% of the anionic lipid POPG.

We synthesized SiPCs to include a fatty acid chain that contained 16 atoms so that we could make a comparison with physiologically relevant model lipid systems, which typically possess fatty acid chains of 16-22 atoms. Siloxane-containing phospholipids **2** and **3** were synthesized using modifications of previously reported literature procedures.(19)(20)

SAXS is sensitive to the presence of MLVs and is used to evaluate the presence of MLVs. Shown in Figure 2 are SAXS data for ~40 mM POPC vesicle suspensions in water as 100 nm ULV (black) and MLVs (gray). For MLVs, positional correlations between the stacked bilayers give rise to a bilayer-bilayer structure factor also known as Bragg peaks at a length scale corresponding to integer multiples of the lamellar repeat distance (e.g., the first Bragg order at $q \sim 0.1 \text{ \AA}^{-1}$, corresponding to a lamellar repeat distance of $\sim 63 \text{ \AA}$). In the ULV sample Bragg peaks are not observed, and vesicles exhibit the typical diffuse scattering for a single lipid bilayer. The inset shows a weighted sum of the black and gray curves as indicated in the inset legend, demonstrating the sensitivity of SAXS to the presence of MLVs.

We are most interested in the scattering regime where bilayer structure (as opposed to vesicle sphericity) dominates the form factor (we get information about vesicle size from the DLS). The scattered intensity of a dilute vesicle suspension is given by:

$$I(q) \propto \frac{S(q) |F(q)|^2}{q^2} \quad (1)$$

where $S(q)$ is the inter-particle structure factor (equal to unity in the infinite dilution limit), $F(q)$ is the form factor. $F(q)$ contains information about the distribution of matter in the

bilayer, more specifically it characterizes the electron density distribution. Mathematically $F(q)$ is the Fourier Transform of the electron density distribution.

The functional model chosen to describe the electron density of the siloxane phospholipids is the three Gaussian model as outlined by Pabst *et al.*(22) In this model the electron distribution is the summation of the head groups, described by a single Gaussian (in total 2, one for each leaflet of the bilayer) and a Gaussian representing the hydrocarbon chains, Eqn 2.

$$\rho(z) = [\rho_H - \rho_w] \left(\exp\left(-\frac{(z - z_H)^2}{2\sigma_H}\right) + \exp\left(-\frac{(z + z_H)^2}{2\sigma_H}\right) \right) + [\rho_C - \rho_w] \exp\left(-\frac{z^2}{2\sigma_C}\right) \quad (2)$$

The position of the Gaussian peak is at z_i where $i=H$ or C and $z_C=0$, with a standard deviation of σ_i . For simplicity, we will refer to $\rho_H - \rho_w$ and $\rho_C - \rho_w$ as $\overline{\rho_H}$ and $\overline{\rho_C}$ respectively. The form factor of this electron density model can then be calculated analytically by applying Eqn 3:

$$F(q) = \int \rho(z) \exp(iqz) dz \quad (3)$$

which yields:

$$F(q) = 2\sqrt{2\pi}\sigma_H\overline{\rho_H} \left(\exp\left(-\frac{(\sigma_H q)^2}{2}\right) \cos(qz_H) + \sqrt{2\pi}\sigma_C\overline{\rho_C} \left(\exp\left(-\frac{(\sigma_C q)^2}{2}\right) \cos(qz_C) \right) \right) \quad (4)$$

Only the cosine terms remain due to the centrosymmetric nature of a single component lipid bilayer.

A benefit of this method is that structural parameters can be derived from simple geometric relationships, without the need of volumetric data. Typically area per lipid A_L is one of the more desirable structural parameters one wants to extract from the data. To begin, we must derive the transverse structure of the bilayer (along the bilayer normal), such as the hydrocarbon length (d_C) and the head group thickness (d_H). Simply, d_H can be estimated from the full width at half maximum (FWHM) of the Gaussian describing the head groups. Using d_H , d_C can be determined as:

$$d_C = z_H - \frac{d_H}{2} \quad (5)$$

Furthermore, the bilayer thickness (dB) is:

$$d_B = z_H + \frac{d_H}{2} \quad (6)$$

Equipped with the transverse structural parameters, we can now tease out the higher dimensional parameters such as A_L and V_C . A_L is:

$$A_L = \frac{1}{\rho_W (\rho_H / \rho_C)} \left(\frac{\rho_H / \rho_C n_C^e}{d_C} - \frac{n_H^e}{d_H} \right) \quad (6)$$

where n_C^e is the number of hydrocarbon electrons and n_H^e the number of head group electrons, respectively. Finally, the volume of the lipid can be calculated by:

$$V_L = A_L \frac{d_B}{2} \quad (7)$$

Because the volume of the PC head group is well characterized and known to be invariant with respect to the phase and temperature(26) of the lipid, the hydrocarbon volume (V_C) can be determined by subtracting $331 \text{ \AA}^3$.

Qualitative inspection of the SAXS curves for 1,2-SiPC and 1,3-SiPC (Figure 3) show a characteristic bilayer form factor without the presence of a bilayer-bilayer structure factor (Bragg peak). The characteristic signature of a structure factor is demonstrated in Figure 2. The spontaneous formation of a unilamellar moiety is in contrast to most phosphocholine phospholipids, which exhibit attractive inter-bilayer forces yielding spontaneous formation of MLVs (Figure 2). The validity of this assumption is demonstrated by the phospho-choline (PC) head group volume remaining constant irrespective of the temperature, lipid phase or chain composition.(24),(25) The experimental and modelled SAXS curves for POPC are presented in Figure 3A.

A comparison of the SAXS data for POPC and the SiPCs reveals a difference in the signal-to-noise ratios for the three sets of data. This difference in the signal-to-noise ratios for the SAXS data corresponding to POPC versus that of the siloxane-phosphocholine species is likely the result of the smaller contrast between the SiPCs and water ($\overline{\rho_c}$, Fehler! Verweisquelle konnte nicht gefunden werden.), with the POPC providing a greater contrast to water, thus resulting in a lower signal-to-noise ratio in the data.

Although this model has previously been validated using both Bragg and diffuse scattering, (22),(26) we checked the model and instrument quality using only the diffuse scattering of the prototypical phospholipid POPC, which also served as a phospholipid control. The optimized POPC fit parameters from our analysis,

Table 1, are in excellent agreement with the values determined by Pabst *et al.*(22) Furthermore, our geometrically derived parameters (Table 1) are in agreement with the high-

resolution structural Scattering Density Profile (SDP) model determination of Ku erka *et al.* (27) For example, we determined the hydrocarbon length (d_C), the head group-head group distance (d_{HH}) and the bilayer thickness (d_B) of POPC to be 14.4 Å, 36.3 Å, and 43.7 Å respectively. For the same parameters, d_C , d_{HH} , and d_B , the SDP analysis yielded 14.6 Å, 37.4 Å, and 39.8 Å.(25) Interestingly, our derived A_L (58.9 ± 2 Å²) is in reasonable agreement with the SDP model derived (62.7 ± 1 Å²) and the geometrically determined hydrocarbon volume is within 5% of the experimentally measured volume for PO chains.(20)

Fit parameters and the geometrically derived structural parameters for SiPCs are summarized in Table 1. The 3-Gaussian model allows for a reasonable estimate of the volume of the hydrocarbon chains to be determined without secondary measurements (Figure 4). Volumetric measurements using conventional densitometry protocols were not possible given the limited quantity of SiPC lipid produced.(20) The derived lipid volume of 1,2-SiPC and 1,3-SiPC were 1574 Å³ and 1577 Å³ respectively, a factor of 7 larger than the volume from the atomic covalent radii. The large volume determined for the SiPCs implies significant disorder in the bilayer core and is very comparable with the volume determined for 1,2-diphytanoyl-*sn*-glycero-3-phosphatidylcholine (diPhyPC).(20) This is not altogether surprising given the similarity in geometry of the trisiloxane moieties and phytanyl chains. However, our derived A_L are lower for the SiPC than diPhyPC (78 Å²) and are more in line with the A_L of a polyunsaturated fatty acid-containing phosphocholines.(28) Liposome size was determined by DLS (Figure 5) and is summarized in Table 1. One size population was observed for POPC ULVs, having an average diameter of 98.1 nm. Two populations were observed for both of the SiPC liposomal preparations examined with the most populous vesicle diameter being 193.7 nm and 123.4 nm for 1,2-SiPC and 1,3-SiPC respectively. Our SAXS data analysis did not reveal the presence of any other type of lipid aggregate suggesting that the less abundant large species are also unilamellar vesicles. The diameter standard deviation (σ) for the vesicles (most abundant population) was below 20% of the diameter for liposomes of 1,2-SiPC and 1,3-SiPC, thus implying very low polydispersities of 0.16 and 0.19 respectively. The unilamellar nature of the vesicles spontaneously formed by the SiPCs, and their relative sizing was confirmed by TEM (Figure 6).

The formation of ULVs from phosphocholine-based systems is unusual. Typically, phosphocholine-based ULVs require multiple extrusion cycles through a membrane with a given porosity to achieve ULVs with a particular diameter (e.g., 100 nm).(6) However, with the addition of a trisiloxane moiety to the end of each of the phospholipid tails, the phosphocholine derivatives formed ~150 nm ULVs following a freeze-thaw cycle and simple agitation. We believe that it is the rather unique properties of siloxanes that are responsible for this phenomenon. Silicones themselves are quite hydrophobic in nature.(30)–(33) In addition, siloxane species have also demonstrated oleophobic tendencies.(34)–(39) It is postulated that the combination of these two properties of siloxanes results in the spontaneous formation of ULVs from the SiPCs. Further detailed studies using a library of siloxane-containing phosphocholine compounds are currently perceived in our laboratory to fully address interactions between SiPC membranes. Included in this library of compounds will be the synthesis of unsymmetrical siloxane-containing phosphocholine derivatives to further explore the influence of the siloxane units on self-assembly and vesicle stability.

Conclusions

We have examined the bilayer properties of vesicles produced from two siloxane-containing phosphocholines. Through SAXS, liposomes of SiPCs bear a remarkable similarity to unsaturated phospholipids. Resulting from the presence of the trisiloxane moiety the area per lipid and lipid volume values are slightly larger than physiologically relevant phosphocholines such as POPC despite the similar bilayer structure.

Liposomes were prepared using only freeze-thaw cycles to give low dispersity suspensions and we feel that these properties will potentially lend these lipids and their liposomes as components of drug delivery preparations. Efforts are currently under way to expand the library of siloxane-containing phospholipids to include various head groups, substitution patterns, and fatty acid chain lengths, and to optimize the preparation of vesicles.

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Abbreviations

diPhyPC	1,2-diphytanoyl- <i>sn</i> -glycero-3-phosphatidylcholine
DLS	dynamic light scattering
FA	fatty acid
FWHM	full width at half maximum
GUV	giant lamellar vesicle
MLV	multilamellar vesicle
PC	phosphocholine
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -3-glycercophosphocholine
POPG	1-palmitoyl-2-oleoyl- <i>sn</i> -3-glycercophosphoglycerol
SAXS	small-angle X-ray scattering
SDP	scattering density profile
SiPC	siloxane-containing phos-phocholine
ULV	unilamellar vesicle

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Figure 1.
 Examples of silicon-containing phosphocholines.(18)–(20)

Figure 2.
The SAXS profiles of POPC MLVs and ULVs and the sensitivity of SAXS to MLV concentration.

Figure 3. SAXS data for ULVs of (A) POPC, (B) 1,2-SiPC, and (C) 1,3-SiPC. The insets show the electron density profiles as a function of distance from the centre of the bilayer.

Figure 4. Electron density profile model $\rho(z)$ as a function of distance from the bilayer centre (z). Key transverse structural parameters are represented.

Figure 5.

Particle size distribution, determined by DLS, of POPC (black dotted line), 1,2-SiPC (blue solid line), and 1,3-SiPC (green solid line) liposomes as aqueous suspensions. POPC ULVs contain 5 mol% of POPG and were extruded through a 100 nm polycarbonate membrane. SiPC suspensions were not extruded. The inset shows representative autocorrelation functions for each species.

Figure 6.
TEM image of spontaneously formed vesicles from 1,2-SiPC.

Table 1

Structural parameters derived from experimental SAXA and DLS data.

Fit Parameters			
	POPC	1,2-SiPC	1,3-SiPC
$\overline{\rho_H}(\text{e}/\text{\AA}^3)$	0.1609 ^a	0.1609 ^a	0.1609 ^a
$\overline{\rho_C}(\text{e}/\text{\AA}^3)$	-0.152±0.002	-0.106±0.002	-0.101±0.003
Z_H (Å)	18.16±0.08	18.2±0.05	17.6±0.1
σ_H (Å)	3.77±0.06	4.6±0.1	4.3±0.2
σ_C (Å)	6.9±0.2	14.1±0.2	13.3±0.3
Geometrically Derived Parameters			
	POPC	1,2-SiPC	1,3-SiPC
d_H (Å)	7.5±0.2	9.1±0.3	8.6±0.5
d_C (Å)	14.4±0.1	9.9±0.5	13.2±0.2
d_B (Å)	43.7±0.2	45.5±0.3	43.9±0.4
d_{HH} (Å)	36.3±0.1	36.3±0.1	35.3±0.1
A_L (Å ²)	58.9±2	69±2	72±4
V_C (Å ³)	961±27	1243±42	1246±81
V_H (Å ³)	331 ^a	331 ^a	331 ^a
DLS ^b Parameters of Liposomes formed by POPC and the SiPCs			
	POPC	1,2-SiPC	1,3-SiPC
Dia. (nm)	98.1	193.7	123.4
σ (nm)	10.6	31.9	23.5
Rel. pop.	100%	79%	89%
Dia. (nm)	-	949.8	684.9
σ (nm)	-	62.0	62.1
Rel. pop.	-	21%	11%

^aFixed using experimentally determined volume(24)^bDynamic light scattering

V = lipid volume

 d_H = head group thickness d_C = hydrocarbon length d_B = bilayer thickness d_{HH} = head group-head group distance A_L = area per lipid