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DiPasquale, Mitchell; Nguyen, Michael H.L.; Castillo, Stuart R.; Heberle, Frederick A.; and Marquardt, Drew. (2022). Identifying Membrane Lateral Organization by Contrast-Matched Small Angle Neutron Scattering. *Methods in Molecular Biology*, 2402, 163-177.
https://scholar.uwindsor.ca/chemistrybiochemistrypub/289

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Identifying Membrane Lateral Organization by Contrast-Matched Small Angle Neutron Scattering

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Abstract

Lipid domains in model membranes are routinely studied to provide insight into the physical interactions that drive raft formation in cellular membranes. Using small angle neutron scattering, contrast-matching techniques enable the detection of lipid domains ranging from tens to hundreds of nanometers which are not accessible to other techniques without the use of extrinsic probes. Here, we describe a probe-free experimental approach and model-free analysis to identify lipid domains in freely floating vesicles of ternary phase separating lipid mixtures.

Keywords— Small angle neutron scattering, Contrast-matching, Lipid rafts, Domains, Phase separation, Lipid vesicles

1 Introduction

The prospect of biological membranes laterally organizing into compositionally and structurally distinct domains has tantalizing implications for a myriad of biological processes [1–3]. Despite vast indirect evidence supporting such membrane functionality, direct evidence of lipid rafts *in situ* have been historically elusive. Recent technological advancements have yielded some of the most compelling evidence to date [4, 5], yet short lifetimes and small length scales render few biophysical techniques capable of resolving these structures.

To reduce analytical difficulties, ternary model membrane systems composed of high-melting (saturated) lipid, low-melting (unsaturated) lipid and cholesterol are commonly implemented to mimic the phase separation phenomenon. At a given composition and environment, the lipid mixture laterally separates into a saturated lipid and cholesterol rich liquid ordered (L_o) phase and an unsaturated rich and cholesterol depleted liquid disordered (L_d) phase. Established phase diagrams for a range of ternary and quaternary lipid mixtures provide a valuable foundation to further research raft-like

lipid domains [6–11]. There is no debate to the organizational complexities that exist between lipids and proteins within cell membranes. The inability of model membrane systems to recapitulate these transient events is often the premise of criticism. In fact, current model membranes may not fully epitomize the size and lifetime of lipid rafts. However, studying the passive, thermally equilibrated domains of simple lipid mixtures provides fundamental insight to the changes in physical chemistry that drive lateral organization. Model membrane studies have been central to understanding factors and interactions that dictate the formation, structure, composition, and stability of lipid domains [12–14].

Fluorescence-based techniques have been at the forefront of the majority of phase separation studies. Phase separated lipid vesicle systems in the micrometer and nanometer range can be characterized by fluorescence microscopy [8] and Förster resonance energy transfer [12, 14, 15], respectively. While these techniques are valuable, there is a prevalent lack of information in length scales ranging from tens to hundreds of nanometers. Though modern advances in super resolution microscopy [16–18] extend the technique to the nanometer range, the requirement of fluorescent probes inherently perturbs the membrane composition and can further modify phase separation by photooxidation [19–21].

Small angle neutron scattering (SANS) provides the ideal tool to interrogate intermediate length scales with the added benefit that extrinsic probes are not required. In an effort to improve accessibility, we spare the intricacies of theory to favour an application perspective [22]. Neutrons are keen to isotopic variation, most notable is the pronounced difference in scattering length between hydrogen's isotopes protium and deuterium. The abundance of hydrogen in organic samples provides a natural avenue to labelling by isotopic substitution. Observing the structure of a typical phospholipid in Figure 1, there are three distinct hydrogen containing regions: acyl chains (yellow), headgroup (green), and surrounding aqueous environment (blue).

[Fig 1 near here]

Neutron scattering intensity is proportional to the contrast ($\Delta\rho$) provided by differing scattering length densities (SLD, ρ) within the sample volume. SLD is a function of molecular volume and coherent scattering length, wherein neutron scattering length is an innate property of each contained isotope. Tables of of common lipid scattering lengths, volumes, and SLDs can be found at the end of this chapter. The SLD of any molecule (*see* **Note 1**), can be calculated as the sum of bound coherent scattering lengths (b_c) over molecular volume (V_m):

$$\rho = \frac{\sum_{i=1}^{n} b_{c_i}}{V_m} \tag{1}$$

This equation can be expanded to elaborate on the average SLD of a specific region of a bilayer by calculating the stoichiometrically weighted sum of individual SLDs. Because we are able to manipulate the coherent scattering length of our sample by varying the proportion of protium and deuterium, we can directly manipulate contrast ($\Delta\rho$) within our sample to highlight or suppresses different scattering regions. For lipid vesicles in solution, SANS emerges from three contributions [14, 22, 23]: (i) the mean difference in SLD between the bilayer and solvent ($\bar{\rho}$ - ρ_s); (ii) the radial difference in SLD normal to the bilayer (between headgroup and acyl chain regions, ρ_h - ρ_c); and (iii) the difference in SLD along the membrane plane (phase separation). Figure 2 graphically demonstrates the different regions contributing contrast.

[Fig 2 near here]

To isolate scattering arising from lateral organization, we aim to highlight component (iii) while suppressing components (i) and (ii) using the concept of *contrast variation*. Evidently, the acyl chain region and aqueous environment are the most hydrogen rich and are therefore the most receptive to deuteration. As such, we will vary the system contrast to match the SLD of the headgroup region ρ_h such that:

$$\rho_h = \rho_c = \rho_s = \bar{\rho} \tag{2}$$

We silence (i) by varying the ratio of H_2O/D_2O in the solvent (ρ_s) and (ii) by substituting a proportion of high-melting lipid with a chain perdeuterated equivalent (ρ_c). Based on this contrast-matching scheme (depicted in Figure 3), when lipids are randomly mixed (at high temperatures) there will be a lack of scattering, but segregation of lipids into L_d or L_o clusters/domains (upon cooling) will increase scattering due to lateral contrast in the acyl chain region (component (iii)) [22].

[Fig 3 near here]

As a case study, we will explore the contrast-matching scheme for the ternary lipid system dipalmitoylphosphatidylcholine/dioleoylphosphatidylcholine/cholesterol (DPPC/DOPC/Chol) at a respective mole fraction of 0.375/0.375/0.25 [8]. The contrast match point is the phosphatidylcholine headgroup, which has ρ_h = 0.181 fm/Å^3. To eliminate scattering contributions from the mean difference in SLD, we construct a background solvent of ρ_s = 0.181 fm/Å^3 containing 34.6 % D_2O (65.4 % H_2O). Section 2.2 elaborates on this calculation. Finally, to ensure no radial scattering contributions between ρ_h and ρ_c, we must balance scattering from the mole fractions of cholesterol and low-melting lipid (DOPC) to ρ_c = 0.181 fm/Å^3 by varying the ratio of perdeuterated (dDPPC) and protiated DPPC. Mathematically, the expansion of equations (1) and (2) for this situation becomes

$$\rho_c = \rho_h = \chi_{\text{chol}}\rho_{\text{chol}} + \chi_{\text{DOPC}}\rho_{\text{DOPC}} + \chi_{\text{DPPC}}\rho_{\text{DPPC}} + \chi_{d\text{DPPC}}\rho_{d\text{DPPC}} \tag{3}$$

where χ and ρ represent the mole fraction and SLD of each component, respectively. Since the total mole fraction of DPPC is to be maintained at 0.375, χ_{DPPC} + $\chi_{d\text{DPPC}}$ = 0.375

and the mole fraction of deuterated lipid can be found using:

$$\chi_{d\mathrm{DPPC}} = \frac{\rho_h - \chi_{\mathrm{chol}}\rho_{\mathrm{chol}} - \chi_{\mathrm{DOPC}}\rho_{\mathrm{DOPC}} - (\chi_{\mathrm{DPPC}} + \chi_{d\mathrm{DPPC}})\rho_{DPPC}}{\rho_{d\mathrm{DPPC}} - \rho_{\mathrm{DPPC}}} \tag{4}$$

Substituting in all known values (from the set of tables flanking this chapter) we determine the mole fraction of deuterated DPPC to be approximately 0.282, leaving a mole fraction of 0.093 protiated. To prepare this contrast-matched system, we would therefore combine DPPC/dDPPC/DOPC/Chol at a mole ratio of 0.093/0.282/0.375/0.25. This contrast-matching scheme can be fine-tuned depending on experimental desires, so long as the conditions in **Note 2** are satisfied.

2 Materials

2.1 Small Angle Neutron Scattering Conditions

When devising an experimental plan it is important to consider the capabilities of the neutron scattering instrument that will be used. Refer to **Note 3** to find a suitable instrument near you and to determine its access mechanism.

1. **Accessible Scattering Vector.** The neutron instrument must be able to probe a scattering vector q–range that corresponds to the length scales of the sample. Generally, a q–range of 0.006 Å$^{-1} < q <$ 0.6 Å^{-1} yields information on features in the length scales of 1 to 100 nm. The scattering vector is inversely proportional to length scale following $q = 2\pi/d$, where d is a real space distance. It is best practice to probe a range that includes vesicle diameter at low-q and domain diameter near high-q.

2. **Scattering Vector Resolution.** Measurement resolution and duration are inversely proportional. At the expense of spatial resolution, increasing the wavelength

spread of the incident neutrons ($\Delta\lambda/\lambda \approx 40$ %) permits more rapid measurements due to the increase in neutron flux. Choice of q-resolution depends on the goal of data analysis. Lower q-resolution is suitable for model-independent analysis such as shown here, whereas modelling data requires more precision [14, 24].

3. **Sample Holder Volume.** Samples are loaded into cylindrical quartz cells or demountable cells with a defined path length of 1 mm, though longer path length cells (2 mm) may also be used (*see* **Note 4**). A lipid concentration of 15 mg/ml tends to provide optimal quality data (*see* **Note 5**), and therefore the size of the sample holder defines the minimum sample mass and volume (*see* **Note 6**).

2.2 Contrast-Matched (CM) Water

To satisfy the contrast-matching scheme described in the *Introduction*, the SLD of the water solvent must match the sample average SLD, $\bar{\rho}$. The SLD of the water solvent can be manipulated by varying the proportions of heavy and light water. The mole fractions χ of H_2O and D_2O that are required to meet this condition can be determined by solving the simple weighted average of $\bar{\rho} = \chi_{\mathrm{H_2O}}\rho_{\mathrm{H_2O}} + \chi_{\mathrm{D_2O}}\rho_{\mathrm{D_2O}}$. The SLDs, ρ, of heavy and light water are found in Table 1 at the end of this chapter. Since CM water is a two component solution the condition $\chi_{\mathrm{H_2O}} = 1 - \chi_{\mathrm{D_2O}}$ reduces the problem to a single variable. Always prepare considerably more contrast-matched water than expected. CM water is needed to hydrate each sample, for background measurements, to wet extruders, as well as for any additional dilutions. Batch-to-batch variation is common and can lead to inconsistencies in the analysis (*see* **Note 7**).

3 Methods

3.1 Preparation of Unilamellar Vesicles

The following protocol will produce a 15 mg/ml suspension of unilamellar vesicles in contrast-matched water suitable for neutron scattering studies. Vesicles will be free of artifacts that may originate from osmotic stress or demixing during preparation. Each step can be done in replicate to prepare multiple samples.

1. Following the scheme defined in the *Introduction*, determine the required lipid mole fractions to satisfy the contrast-matching condition.

2. Using a gas-tight glass syringe, combine lipids by transferring required volumes of chloroform stock solutions to a 20 mL flat-bottom scintillation vial of known weight (*see* **Note 8**).

3. Evaporate bulk solvent with a gentle stream of inert gas.

4. Remove trace solvent by drying the lipid to a film *in vacuo* overnight (*see* **Note 9**). To ensure lipids remain mixed and avoid cholesterol crystallization while drying and throughout the preparation [25], maintain the temperature slightly above the melting temperature of the high-melting lipid component, T_m^{high}. For most lipid combinations, $\approx$ 55 °C is sufficient.

5. Using an analytical balance, determine the exact mass of the dried lipid film (considering the mass of the pre-weighed vial).

6. Hydrate the lipid film to 15 mg/ml (*see* **Note 5**) by adding CM water pre-warmed slightly above T_m^{high}. Cap the vial tightly and wrap with parafilm to prevent the cap from loosening.

7. Allow vesicles to form by incubating for one hour in a water bath (*see* **Note 10**) or heat block slightly above T_m^{high}. Intermittently vortex the sample aggressively until

all lipid has detached from the bottom of the vial into a milky suspension of multilamellar vesicles.

8. After the incubation, freeze the sample at -80 °C, thaw in a water bath or heat block above T_m^{high}, then vortex. Repeat this freeze–thaw–vortex cycle five times (*see* **Notes 11** and **12**).

9. Fit a hand-held miniextruder with a polycarbonate membrane of 50 nm diameter pores (*see* **Note 13**). Heat the entire assembly above T_m^{high} on a hot plate. Use a thermometer to ensure the temperature does not greatly exceed T_m^{high} as this can accelerate lipid oxidation and/or damage the extruder apparatus.

10. Prime the extruder and ensure there are no leaks by passing through CM water.

11. Extrude the sample by 31 passes through the polycarbonate membrane. The odd number of passes ensures no multilamellar contamination in the extruded product. Clean and dry the extruder assembly and use a new polycarbonate membrane for each sample. Extruded lipid suspensions often exhibit an opal-like iridescence.

12. Particle sizing measurements, such as dynamic light scattering, can confirm the success of extrusion into a monodisperse population.

13. Samples should be stored cool to discourage lipid oxidation; however, conditions less than 4 °C can freeze deuterated water. Frozen samples need to be re-extruded prior to measurement.

3.2 Small Angle Neutron Scattering

Neutron scattering studies will vary greatly depending on the instrument specifications and capabilities. Always consult with an instrument scientist when designing an experiment. Here, we lay out a general workflow of considerations when conducting measurements at the beam line. As a rule of thumb, high q-resolution data will require

60 minutes per sample collection with three minutes per transmission measurement, though this is directly dependent on neutron flux. Refer back to *Small Angle Neutron Scattering Conditions* in the *Materials* section for an explanation.

1. Fit the sample stage with a temperature-controlled sample environment designed for cylindrical or demountable cells. These multi–position sample blocks often have multiple temperature zones to expedite data collection.

2. Tune the characteristics of the incident neutron beam. These properties, such as wavelength and wavelength spread are optimized for each neutron source and instrument. The instrument scientists will assist in adjusting these parameters for your experimental needs.

3. Collect necessary control measurements including flux, absolute resolution, and blocked beam scattering (cosmic and stray radiation).

4. Measure background scattering from CM water and transmission at each temperature that the samples will be measured. (*see* **Notes 14** and **15**).

5. Load samples into the sample block and allow the temperature to equilibrate (*see* **Note 16**). Start at a cool temperature and incrementally heat the sample between measurements (*see* **Note 17**).

6. Following instrument specific guidelines, correct the two dimensional data for detector pixel sensitivity, dark current, sample transmission, and background scattering from CM water. As samples scatter isotropically, the data are then reduced to one dimensional scattering plots of intensity I against scattering vector q by azimuthally averaging (*see* **Note 18**).

3.3 Data Analysis–The Porod Invariant

Based on the contrast matching scheme, and proved by Heberle et al., lateral segregation is the dominant source of scattering [14]. As such, the degree of phase separation is proportional to the amount of scattering. The integrated total scattering intensity, or Porod invariant Q, can be calculated as [26, 27]:

$$Q = \int q^2 I(q) dq \tag{5}$$

The Porod invariant does not depend on the form of the scattering entity, and so this type of analysis is compatible with data collected using a larger wavelength spread. Collecting data using a "white beam" configuration can dramatically decrease sample measurement times for a higher throughput. From a temperature series, a plot of Q as a function of temperature produces a decay plot that reveals the melting of domains [24]. Samples that demonstrate a smaller decrease in Q with increasing temperature are considered to have more thermally stable domains.

To a first approximation, the Porod invariant is dependent on the domain area fraction (a_{L_o}) and the lateral contrast between the L_o and L_d phases ($\Delta\rho$) following the expression $Q \approx a_{L_o}(1 - a_{L_o})\Delta\rho^2$ [27]. While this analysis is reliable for identifying the presence of domains, it is important to consider that this method cannot directly report on domain area fraction, size, shape, or composition. As a result, it is not possible to decouple the modes of domain melting, being a combination of decreasing area fraction and decreasing contrast. For a deeper insight, including domain sizes, Monte Carlo-based modelling approaches have been used for insight into domain sizes [14, 22, 23]. In this case, vesicles are modelled as a shell of finite thickness populated by clearly defined randomly placed circular patches. Though this approach is valuable, it requires a reliable phase diagram and assumes a number of features, including a homogenous domain composition, sharp interfaces, and uniform morphologies which may not be

valid for all lipid mixtures.

4 Notes

1. Larger molecular volumes often vary with temperature. Therefore, SLD can be considered as a thermally dependent parameter. Since we want to observe the formation of domains at a low-to-intermediate temperature, calculate ρ using a volume above the miscibility temperature such that scattering is suppressed in the absence of domains. Pencer et al. find that phase associated changes in molecular volume can be considered negligible [22].

2. A wide range of additional compounds can be contrast-matched to observe their effect on membrane organization by incorporating their scattering contributions in the contrast-match scheme. To achieve this, in addition to calculating the scattering length, the molecular volume and intuition on its membrane location must be known to properly attribute ρ to the appropriate region. For example, cholesterol is known to reside largely associated with the acyl chains, and so its scattering is encompassed in the acyl chain region ρ_c, but does not affect ρ_h.

3. Visit https://neutronsources.org/ to find a suitable and accessible neutron source.

4. Longer path lengths will decrease sample transmission. A 1 mm path length is best to achieve a transmission greater than 90 % to neglect multiple scattering.

5. A concentration of 15 mg/ml is optimal to maintain an efficiently scattering sample that is sufficiently dilute to avoid contributions from interparticle structure factor.

6. A typical 1 mm cell requires a volume of 300–500 μl.

7. Due to the density fluctuations of water with temperature, we find it most accurate to prepare the H_2O/D_2O mixture directly by mass using an analytical balance.

8. It is best to transfer lipids through a chloroform or 2:1 chloroform:methanol stock solution, depending on the lipid species. Preparing samples from lyophilized powder may produce artifacts from lipid demixing.

9. Failure to completely remove solvent can affect results [28–30]. We advise no less than 6 hours under vacuum to ensure trace solvent removal.

10. If using a water bath, a tight wrapping of parafilm is crucial for preventing the cap from loosening. Contamination from the water bath can perturb the H_2O/D_2O ratio of the CM water and compromise the contrast match.

11. Freeze-thaw cycles assist in decreasing the number of multilayers in the vesicles to facilitate the extrusion step [31]. Ensure the sample is completely freezing/thawing at each point in the cycle.

12. In most cases, so long as the sample is protected from oxidation, it can remain frozen as multilamellar vesicles for an indefinite amount of time. This is an optimal "break point" in the preparation. Once the samples are extruded, they should be measured well within a week.

13. Using a small vesicle size (50 nm diameter) discourages multilamellarity. To study lipid populations greater than 50 nm in diameter, incorporate 5 mol % charged lipid (typically a phosphatidylglycerol headgroup) to electrostatically drive unilamellarity [32]. Include this lipid species in the contrast-matching calculations.

14. For instruments that require multiple configurations to cover the desired scattering vector, transmission measurements can be collected only at the farthest sample-to-detector distance (lowest q) so long as the optics up–beam do not change between configurations.

15. Over small temperature ranges, changes in the incoherent background scattering

from water are minimal. If necessary, for efficient use of beam time CM water background can be collected during temperature equilibrations.

16. Though small sample volumes ($<500\ \mu$l) will change temperature very rapidly, we advise allowing greater than 10 minutes for thermal equilibrium to be achieved.

17. Cooling may result in phase separation artifacts from kinetic trapping [21]. Often times it is wise to collect data from both heating and cooling scans to check for sample hysteresis.

18. Neutron scattering directly provides an ensemble average of $\approx 10^{15}$ vesicles in solution [22], far greater than comparable fluorescence studies.

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5 Figure Captions

Figure 1. The radial components of a typical phospholipid (POPC, in this example) can be divided into three scattering regions that contribute contrast. These regions include (A) the acyl chains (CH_2 and CH_3 groups), (B) the headgroup (two carbonyl group, glycerol, phosphate, and choline), and (C) the aqueous solvent.

Figure 2. Scattering contrasts arise from spatially resolved regions of varying scattering length densities in the bilayer. Here, colours are used to depict different SLDs. To achieve a contrast matching condition, we aim to minimize the difference between the solvent (ρ_s, blue), the headgroup (ρ_h, green), and the acyl chains (ρ_c, yellow) while maximizing the difference between the acyl chains (dark vs light yellow). The components contributing to the average SLD $\bar{\rho}$ are also shown.

Figure 3. Depiction of contrast-matching experiment. At high temperatures lipids are laterally mixed in the plane of the bilayer, yielding a SLD equivalent to the headgroup and solvent; thus, scattering is suppressed due to a lack of contrast. As domains coalesce, scattering arises from lateral contrast between acyl chains in L_o (light green) and L_d(dark green) phases. The green colour signifies that the system SLD reflects that of the headgroup, ρ_h.

Table 1: SLDs of water, cholesterol, and common lipid headgroups. Sources a–f correspond to references [33–38], respectively. Element X refers to a correction for an exchangeable proton. The absolute volume of a lipid can be determined from the sum of the headgroup and acyl chain volumes.

Component	b **[fm]**	$\mathbf{V}_m$ **[Å^3]**	ρ **[fm/Å^3]**
H_2O	-1.68	30.4	-0.055
D_2O	19.15	30.5	0.628
cholesterol *($C_{27}H_{46}O$)*	13.25	630[a]	0.021
PC headgroup *($C_{10}H_{18}NO_8P$)*	60.07	331[b]	0.181
PG headgroup *($C_8H_{10}X_2O_{10}P$)*	92.28	291[c]	0.215
PE headgroup *($C_7H_{12}NO_8P$)*	62.568	245[d]	0.255
PS headgroup *($C_8H_8X_3NO_{10}P$)*	115.789	278[e]	0.417
SM headgroup *($C_9H_{17}X_2N_2O_6P$)*	68.261	274[f]	0.249

Table 2: Scattering lengths, b and acyl chain volumes of common same acyl chain lipids. Italicized data are extrapolated from existing data, or collected following the methods of [35].

	DL dilauroyl 12:0 (d46)	**DM dimyristoyl 14:0 (d54)**	**DP dipalmitoyl 16:0 (d62)**	**DS distearoyl 18:0 (d70)**	**DO dioleoyl 18:1**
b [fm]	-25.782	-29.110	-32.438	-35.766	-20.81
(perdeuterated)	(453.078)	(533.030)	(612.982)	(692.934)	
PC Acyl Volumes [Å^3]					
20 °C	655.3[a]	718.5[a]	812.5[a]	907[b]	958.5[d]
30 °C	660.6[a]	766.6[a]	822.1[a]	917[b]	967.7[d]
40 °C	668.2[a]	777.2[a]	839.3[a]	929[b]	982.6[d]
50 °C	675.5[a]	786.3[a]	896.8[a]	941[b]	993.3[d]
60 °C	682.9[a]	795[a]	908[a]	1017[c]	1005.4[d]
PG Acyl Volumes [Å^3] [e]					
20 °C	654.9	720	815.8	900.4	966.5
30 °C	662.6	766	825.5	912.4	974
40 °C	666.1	775	854.7	922.7	981.8
50 °C	671.4	783	897.8	943.5	990
60 °C	680.5	790	907.1	1014	997.2
PE Acyl Volumes [Å^3]					
20 °C	*626.6*				
30 °C	*629.8*				
40 °C	660.8[f]				
50 °C	669.3[f]	*764*			
60 °C	678.2[f]				
PS Acyl Volumes [Å^3]					
20 °C	*650.7*	*725.5*	*815.9*		*964.1*
30 °C	*660.4*	*734.6*	*825.1*		*973.1*
40 °C	*669.1*	*779.4*	*835.5*		*982.9*
50 °C	*676.6*	*789.9*	*847.7*		*992.4*
60 °C	*683*	*805.2*	*907.1*		*1001.9*

Data found in [a][39] [b][40] [c][41] [d][42] [e][35, 43] [f][36].

Table 3: Scattering lengths, b and acyl chain volumes of common mixed acyl chain lipids. Italicized data are extrapolated from existing data, or collected following the methods of [35].

	PO palmitoyl oleoyl 16:0/18:1	**SO stearoyl oleoyl 18:0/18:1**	**PD palmitoyl docosahexaenoyl 16:0/22:6**	**SD stearoyl docosahexaenoyl 18:0/22:6**
b [fm]	-26.624	-28.288	7.438	5.774
PC Acyl Volumes [Å^3]				
20 °C	916[a]	969[a]	965.6[b]	
30 °C	925[a]	978[a]	975.4[b]	1035.4[b]
40 °C	934[a]	*987.4*	985.1[b]	
50 °C	944.5[a]	996.5[a]	*994.85*	
60 °C	953[a]	1006[a]	*1004.6*	
PG Acyl Volumes [Å^3] [c]				
20 °C	910.9	974.5		
30 °C	917.7	981.8		
40 °C	931.9	989.3		
50 °C	942.7	997.6		
60 °C	952.6	1006.1		
PE Acyl Volumes [Å^3]				
20 °C	*885.7*	*929.8*		
30 °C	925[d]	948.7[d]		
40 °C	935.2[d]	985.3[d]		
50 °C	944.4[d]	996.6[d]		
60 °C	*953.5*	*1006.8*		
PS Acyl Volumes [Å^3]				
20 °C	917.1[e]	*968.9*		
30 °C	923.8[e]	*979.4*		
40 °C	930.4[e]	*989.9*		
50 °C	937[e]	*999*		
60 °C	*943.7*	*1007.9*		
SM Acyl Volume [Å^3] [f]				
	palmitoyl SM d18:1/16:0	**stearoyl SM d18:1/18:0**		
b [fm]	-24.96	-26.624		
45°C	877.6			
55°C	887.7	952.8		
65°C		963.1		

Data found in [a][41] [b][44] [c][35, 43] [d][36] [e][37] [f][38].