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**Complete List of Authors:**  
- Krzyzanowski, Natalie; University of Illinois - Chicago, Physics  
- Porcar, Lionel; Institut Laue-Langevin, Large Scale Structures Group; University of Delaware, Department of Chemical Engineering,  
- Garg, Sumit; Argonne National Laboratory, Materials Science Division; University of Illinois - Chicago, Physics  
- Butler, Paul; National Institute of Standards, Gaitherburg, Center for Neutron Research; University of Delaware, Department of Chemical Engineering,  
- Castro-Roman, Francisco; Centro de Investigacion y de Estudios Avanzados del IPN,  
- Bautista, Pedro; Centro de Investigacion y de Estudios Avanzados del IPN,  
- Perez-Salas, Ursula; University of Illinois Chicago, Physics; Argonne National Laboratory, Materials Science Division

Natalie Krzyzanowski,† Lionel Porcar,b,c Sumit Garg,†d,a Paul Butler,ec Francisco Castro-Roman,f Pedro Jesus Bautista,f and Ursula Perez-Salas*ad

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DOI: 10.1039/In the comment by Epand et al. on our recent article1, it is stated that the term “cholesterol solubility limit” is misused. As Epand et al. point out, there is extensive literature on cholesterol phase separation in phospholipid bilayers and this term is used to define the appearance of cholesterol crystals. Moreover, as they state, this does not preclude the membrane from existing as bilayered crystals or cholesterol-only domains within the membrane itself. Since our SANS data directly measured the maximum amount of cholesterol harboured by POPC and POPS membranes, it may have been more appropriate to use the term “cholesterol saturation limit”. Nonetheless, we stated that the saturation and solubility limits of cholesterol coincide in both POPC and POPS. Epand and et al. suggest that the data shown was insufficient to uphold this claim. Herein, we present data that supports the coincidence of cholesterol’s saturation limit with cholesterol’s solubility limit in 100nm POPS unilamellar vesicles, where previously it has been reported to not be the case.2

Although the phase diagram of mixtures of lipids and cholesterol can generally be described by results from a mean field approach3, experimentally it is known that lipid saturation plays a major role in phase behavior. For example, with unsaturated lipids, the liquid ordered phase is absent4, 5. Consequently, cholesterol in an unsaturated lipid membrane remains in a single (condensed) fluid phase until a cholesterol-only phase emerges. This is cholesterol’s solubility limit. In the model by Pata et al.3, above this cholesterol solubility limit, the cholesterol-only phase forms solely outside the membrane as crystals. However, whether the membrane can actually harbor cholesterol above the solubility limit in the form of cholesterol-only domains within the membrane remains contentious. Experiments and MD simulations using saturated lipids and cholesterol show that the membrane can retain cholesterol above the solubility limit in the form of cholesterol-only bilayered domains6.7. For unsaturated lipids, however, this may not be the case. POPC, for example, cannot retain cholesterol above the solubility limit in the membrane8.9. On the other hand, POPS has been reported to retain cholesterol-only domains in the membrane’s bilayer up to 66 mol% cholesterol2, which is well above a solubility limit of 36 mol%10, 11. In our small angle neutron scattering (SANS) study we directly showed that in 100nm unilamellar lipid vesicles of POPS with cholesterol, the bilayer retains a relatively high concentration of cholesterol (~70 mol%). To determine whether cholesterol-only domains are present in these vesicles above the reported solubility limit of ~36 mol% we collected small and wide angle x-ray scattering (SAXS and WAXS) data on samples with increasing amounts of cholesterol and shown in Figure 1A. The X-ray data show no evidence of the 17 Å and/or 34 Å anhydrous cholesterol peaks6.8, 11. Additionally, we took differential scanning calorimetry measurements for the 50 mol% cholesterol sample. The resulting heating and cooling scans are shown in Figure 1B and do not show an anhydrous cholesterol peak as detected previously10, 11.
SANS is an exquisitely sensitive technique in detecting compositionally distinct coexisting regions or domains in membranes. If the scattering length density of the solvent, $\rho_{\text{solvent}}$, matches that of the average scattering length density of the whole membrane as if it was fully mixed ($\langle \rho_{\text{vesicle}} \rangle$), then any excess scattering, from an otherwise flat curve, is solely due to the presence of compositional inhomogeneities within the membrane. SANS data for 100nm unilamellar vesicles of deuterated POPS with 60 mol% cholesterol in a solvent that matches $\langle \rho_{\text{vesicle}} \rangle$ is shown in Figure 2A. The data shows some remaining contrast between the lipid tails and headgroups as expected, but the signature of domains is missing. Epand et al. argued correctly in their comment that if cholesterol domains were sufficiently small, there would be no excess scattering. Yet, at what domain size can we consider cholesterol to be in cholesterol-only domains as opposed to being mixed in the membrane? Figure 2A shows SANS data of a contrast matched 1:1 deuterated DPPC:DLPC mixture with 12 mol% cholesterol which had been concluded to be fully mixed using FLRRT (to within the resolution of 5nm of the fluorescent pair used for this measurement). Indeed, when domains are small (~5nm), the excess scattering is not large as compared to data from DPPC:DLPC (devoid of cholesterol) where domains are of order ~30nm (Figure 2B). However, clearly the signal from 5nm domains is sufficient to show that, in the case of 60 mol% cholesterol in POPS, there is no evidence of domains. Consequently we conclude that we do not detect cholesterol-only domains in unilamellar vesicles of POPS, setting the cholesterol solubility limit in POPS to that reported in our recent publication.

As a final comment we want to reiterate that MD simulations have been able to model, in saturated lipid systems, phase separation of ordered phases and the formation of cholesterol-only bilayered domains above the solubility limit, and are therefore consistent with experiments. Similarly, our MD simulations for POPC and POPS agree with our experimental results except that beyond the saturation limit, cholesterol is placed in the center of the membrane instead of being expelled from the bilayer to form crystals. While recent all-atom simulations on pure cholesterol bilayers appeared to show a single stable bilayered cholesterol-only domain, this behavior is inconsistent with what is observed experimentally, where, independent of saturation, the membranes’ cholesterol saturation limit is ~70 mol% or less. Thus coarse-grained and all-atom MD simulations are still unable to capture the behavior of cholesterol in membranes beyond the saturation limit.

In conclusion, even though Epand et al. correctly state in their comment that the SANS data presented in our previous study cannot truly exclude the existence of cholesterol-only domains, the new data presented in this response clearly support our previous conclusions. As a result, we thank Epand et al for their criticism and feedback.

Figure 1. A) X-ray diffraction data plotted as a function of intensity versus amplitude of the momentum transfer vector $q$. Powdered samples of POPS and cholesterol were measured at room temperature and data are shown as the bottom two spectra. 100nm unilamellar vesicles of POPS with 0, 25, and 50% of molar cholesterol were measured at 25°C (solid lines) and 63°C (thicker dashed lines). The cholesterol anhydrous peaks, marked using arrows, are clearly not observed in the XRD data for the vesicles. B) µDSC heating and cooling scans, offset for viewing clarity, for a single sample of 100nm unilamellar vesicles composed of POPS and Cholesterol in a 1:1 molar ratio dissolved in water. The amount of cholesterol was 17 mg with 33 mg of POPS. The four top curves are heating scans and the bottom four curves are cooling scans. The numbers denote the order in which scans were run. All curves were obtained at a 1°C/min scan rate except for curves 7 and 8 which were run at 0.5°C/min.
Notes and references

a Physics Department, University of Illinois at Chicago, Chicago, IL 60607, USA.
b Large Scale Structure Group, Institut Laue-Langevin, Grenoble F-38042, France
c Department of Chemical Engineering, Colburn Laboratory, University of Delaware, Newark, Delaware
d Materials Science Division, Argonne National Laboratory, Lemont, Illinois
e Center for Neutron Research, National Institute of Standards and Technology, Gaithersburg, Maryland, MD 20899, USA
† Present address: Berg LLC, Framingham, MA 01701, USA


Figure 2. A) SANS measurements for contrast matched unilamellar vesicles of two samples: d31-POPS with 60 mol% cholesterol (circles) and d62-DPPC:DLPC 1:1 with 12 mol% cholesterol (triangles). The SANS curves for the dDPPC:DLPC sample were shifted in intensity so that the incoherent backgrounds, which depend on solvent composition, would be aligned for the two systems. The dDPPC:DLPC:Cholesterol data is shown above and below the melting temperature. The solid blue line corresponds to a vesicle form factor fit to the dPOPS:Chol data at 20°C. B) SANS measurements above and below the melting temperature for contrast matched 50nm unilamellar vesicles of d62-DPPC:DLPC 1:1. The fits for the two temperatures correspond to the form factors of the bead models‡‡‡‡ representing two phases in the membrane (unpublished data).


100 nm unilamellar vesicles of POPS

- 0 mol% cholesterol
- 50 mol% cholesterol

Powdered samples

- Cholesterol

$I(q)$ [cm$^{-1}$]

$q$ (Å$^{-1}$)

34 Å

17 Å

304x283mm (96 x 96 DPI)