



Reply to the 'Comment on "Cholesterol Solubility Limit in Lipid Membranes probed by Small Angle Neutron Scattering and MD simulations"' by R. Epanand, *Soft Matter*, 2015, 11, DOI:10.1039/C4SM02819H

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ARTICLE TYPE

Reply to the ‘Comment on “Cholesterol Solubility Limit in Lipid Membranes probed by Small Angle Neutron Scattering and MD simulations”’ by R. Epanand, *Soft Matter*, 2015, 11, DOI: 10.1039/C4SM02819H

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DOI: 10.1039/ In the comment by Epanand et al. on our recent article¹, it is stated that the term “cholesterol solubility limit” is misused. As Epanand 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 them 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. Epanand 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 case².

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 absent^{4, 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 domains^{6, 7}. For unsaturated lipids, however, this may not be the case. POPC, for example, cannot retain cholesterol above the solubility limit in the

membrane^{8, 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 peaks^{6, 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 previously^{10, 11}.

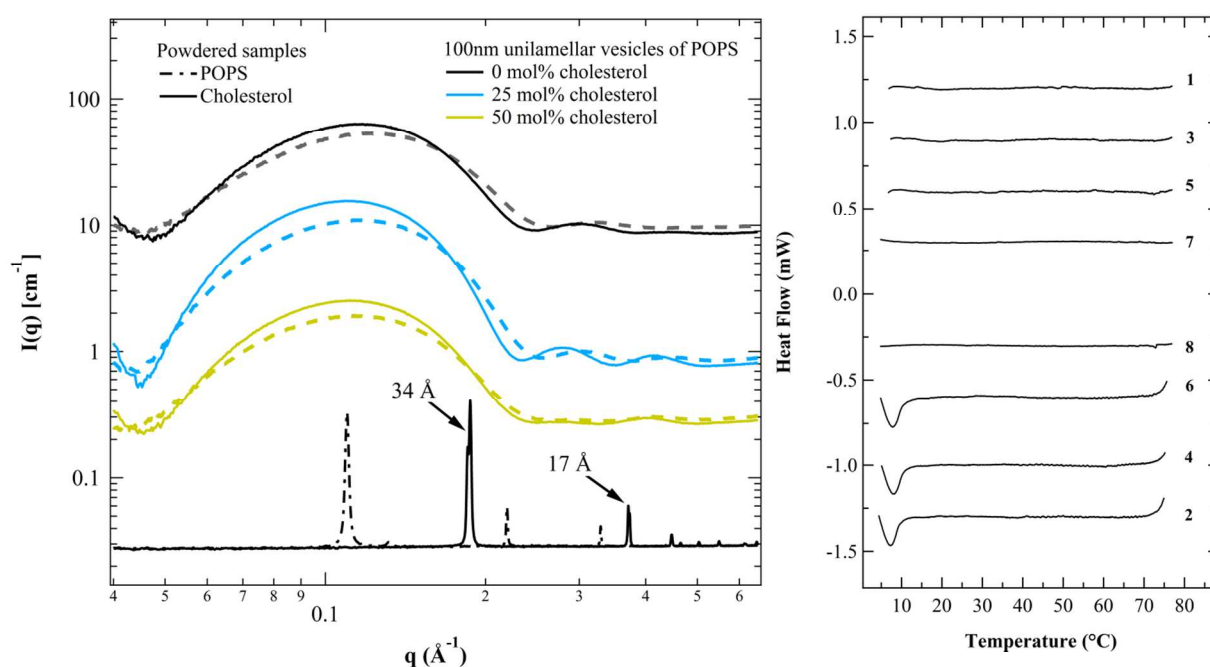


Figure 1. A) X-ray diffraction data plotted as a function of intensity versus amplitude of the momentum transfer vector q . Powdered samples of POPS and cholesterol were measured at room temperature and data are shown as the bottom two spectra. 100nm unilamellar vesicles of POPS with 0, 25, and 50% of 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 $1^{\circ}\text{C}/\text{min}$ scan rate except for curves 7 and 8 which were run at $0.5^{\circ}\text{C}/\text{min}$.

SANS is an exquisitely sensitive technique in detecting compositionally distinct coexisting regions or domains in membranes¹². If the scattering length density of the solvent, ρ_{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 expected¹³, 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 FRET (to within the resolution of 5nm of the fluorescent pair used for this measurement)¹⁴. Indeed, when domains are small ($\sim 5\text{nm}$), the excess scattering is not large as compared to data from DPPC:DLPC (devoid of cholesterol) where domains are of order $\sim 30\text{nm}$ (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 publication¹.

As a final comment we want to reiterate that MD simulations have been able to model, in saturated lipid systems, phase separation of ordered phases¹⁵ and the formation of cholesterol-only bilayered domains above the solubility limit, and are therefore consistent with experiments^{7, 16}. 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 domain¹⁷, this behavior is inconsistent with what is observed experimentally, where, independent of saturation, the membranes' cholesterol saturation limit is $\sim 70\text{ mol}\%$ 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.

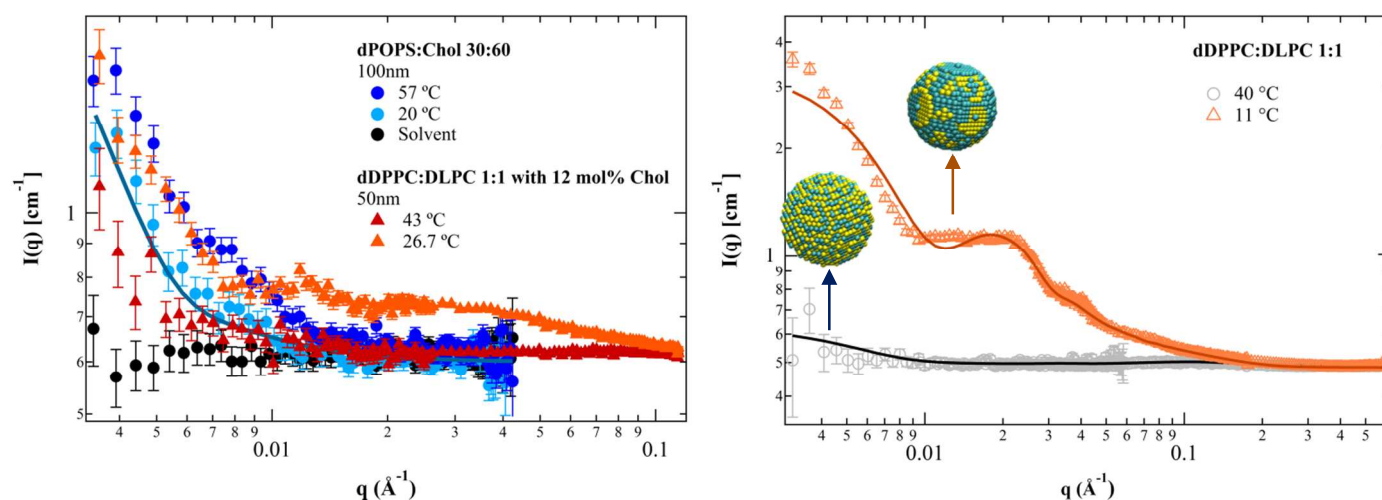


Figure 2. A) SANS measurements for contrast matched unilamellar vesicles of two samples: d31-POPS with 60 mol% cholesterol (circles) and d62-DPPC:DLPC 1:1 with 12 mol% cholesterol (triangles). The SANS curves for the dDPPC:DLPC sample were shifted in intensity so that the incoherent backgrounds, which 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).

Notes and references

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[†] Electronic Supplementary Information (ESI) available. See DOI: 10.1039/b000000x/

[‡] http://avantlipids.com/index.php?option=com_content&view=article&id=1600&Itemid=381

^{‡‡} X-ray diffraction measurements of powder samples were performed at the Life Sciences Collaborative Access Team (LS-CAT) at the Advanced Photon Source (APS). Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan

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³⁵ ^{††††} SANS measurements were performed on D22 at the Institut Laue-Langevin (ILL) in Grenoble France. Data reduction using GRASP : <http://www.ill.eu/en/html/instruments-support/instruments-groups/groups/lss/grasp>; Data analysis using IGOR (S. R. Kline. 2006. Reduction and analysis of SANS and USANS data using IGOR Pro. J Appl. Cryst. 39(6):895) and SANSVIEW: <http://danse.chem.utk.edu/sansview.html>

^{†††††} Bead modeling and fitting with MONSA (D.I. Svergun (1999) Restoring low resolution structure of biological macromolecules from solution scattering using simulated annealing. *Biophys. J.* **76**, 2879-2886)

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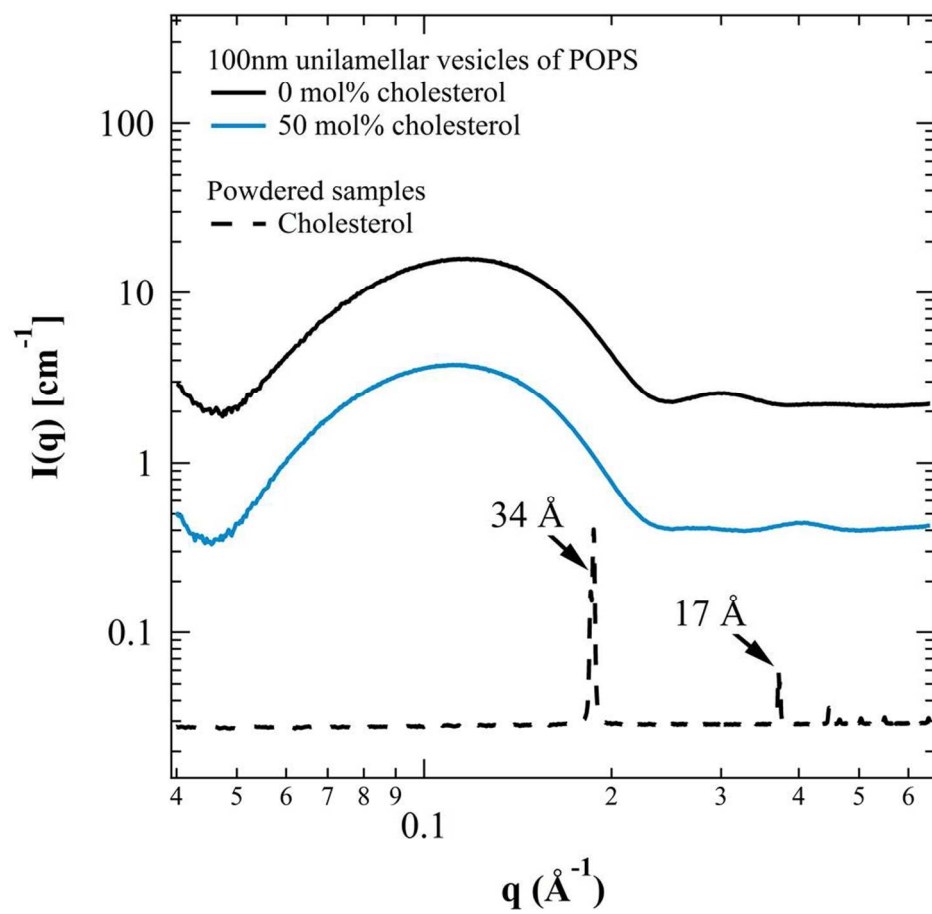
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