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Response to “Reply to the 'Comment on "Cholesterol Solubility Limit in Lipid Membranes probed by Small Angle Neutron Scattering and MD Simulations by R. Epand, Soft Matter. 2015,11.: DOI: 10.1039/C4SM02819H" by Natalie Krzyzanowski, Lionel Porcar, Sumit Garg, Paul Butler, Francisco Castro-Roman, Pedro Jesus Bautista and Ursula Perez-Salas'’’

As authors of the “Comment on ‘Cholesterol Solubility Limit in Lipid Membranes probed by Small Angle Neutron Scattering and MD simulations’”, we wish to comment on both the form and content of the Reply cited above [1]. In their original article [2], Garg *et al.* used neutron scattering techniques to determine the limiting amount of cholesterol which vesicles of either POPS or POPC can accommodate. They called this amount ‘‘the cholesterol solubility limit’’. Since the solubility limit ofcholesterol in lipid membranes has long been defined in the literature as the concentrationat which cholesterol phase separation and crystallite formationbegin to take place, we found that the report of Garg *et al.* [2] could be easily misinterpreted by the community and definitely requires some clarification, as indicated in our Comment.

To justify their use of the term “cholesterol solubility limit”, (for this, they have now substituted the term “saturation limit”), the authors presented new measurements, which, in our opinion, are inaccurate. In particular, while it is known that the pseudobilayer periodicity (34 Å) of cholesterol can only be readily detected by X-ray diffraction upon cholesterol phase separation in multi-lamellar vesicles [3], the authors look for it in an X-ray diffraction pattern from a sample of unilamellar phospholipid/cholesterol vesicles (Ref. 1, Fig. 1A). For evidence of phase separation in single phospholipid bilayers, only in-plane periodicities of cholesterol crystals can be observed [4]. Furthermore, details of the DSC instrumentation used by Krzyzanowski *et al.* (Fig. 1B), to be able to evaluate the sensitivity of the measurement, are lacking. This is a particular problem with mixed PS/cholesterol unilamellar vesicles, since the peak height of the polymorphic transition of anhydrous cholesterol at 37^{o} C may be much smaller than in the case of multi-lamellar vesicles [3]. In addition, it is not clear why there is an exotherm in the cooling scans at about 7^{o}C, that is not seen in heating scans or why this transition disappears completely

when the scan rate is decreased by only two fold. Even more important is the authors' claim in the abstract of their Reply that "the saturation and solubility limits of cholesterol coincide in both POPC and POPS". We fail to see evidence for this in their new data. No measurements at all are provided for POPC/cholesterol mixtures. In addition, the saturation limit for cholesterol in POPS unilamellar vesicles is presented as 73+/-3 mol% [1] while in their Reply in order to determine the solubility limit they do not present data for molar ratio of cholesterol/phospholipid above 1:1.

In summary, we emphasize that determination of cholesterol solubility in phospholipid mixtures is a multi-faceted experimental task, requiring attention both to the choice of the appropriate measurement technique and to the proper material preparation protocol. Such an effort cannot be properly and justifiably included in a brief note.

References

[1] Krzyzanowski *et al*., Soft Matter, 2015, **11**, 5582.

[2]Garg *et al.,*Soft Matter, 2014, **10**, 9313.

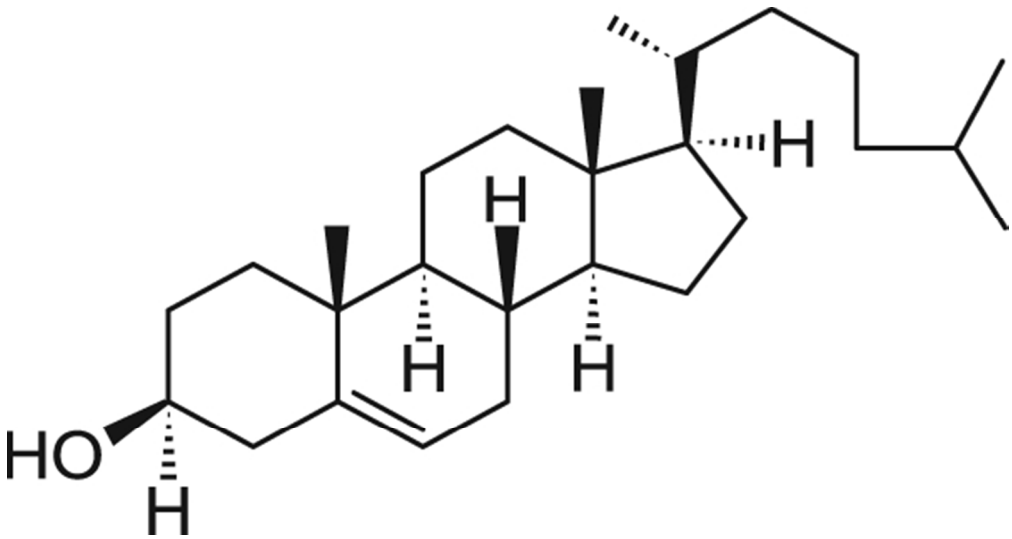
[3] Epand*et al.,* Biophysical Journal, 2000,**78**, 866.

[4] Ziblat*et al.,* JACS, 2010, **132**, 9920.

Richard M. Epand

Diana Bach

Ellen Wachtel



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Cholesterol