REPLY TO NAGLE ET AL.:

The universal stiffening effects of cholesterol on lipid membranes

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Based on neutron spin-echo (NSE), solid-state NMR, and molecular dynamics (MD) simulations, we report clear evidence that cholesterol stiffens 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) membranes (1). Contrary to statements by Nagle et al. (2), the relaxations measured by NSE and NMR directly relate to elastic membrane constants (3, 4). While both NSE and NMR spectroscopy measure the time-averaged τ-decay, $\langle A(t)A(t+\tau)\rangle$, the data analysis methods separate the mean-square amplitude (MSA) from membrane dynamics, providing information about equilibrium and dynamical bilayer properties (4). In the case of isotropic systems (simplest case), the MSA $= \langle A(t)^2\rangle$ and the time average $\langle A(t)\rangle$ is zero. However, for ordered systems such as lipid bilayers, the time average is nonzero [i.e., $\langle A(t)\rangle \neq 0$], and consequently the MSA $= \langle A(t)^2\rangle - \langle A(t)\rangle^2$ (4). Hence, the time interval becomes crucial to interpreting both spectroscopic and scattering experiments.

If only local motions are considered, the MSA corresponds to the segmental order parameter S_{local} averaged over local degrees of freedom (DOFs). For hierarchical dynamics captured by NMR relaxometry and NSE, the MSA for the most rapid motions depends on the residual interaction that remains from averaging the local DOF. The residual DOFs then yield the MSAs for the next slower motions, and so on until the limit of isotropic averaging is reached (4). In NMR spectroscopy, equipartition among a continuum of first-order relaxation modes gives a characteristic $\nu^{-1/2}$ frequency signature scaled by $\langle A(t)\rangle^2_{\text{local}} \propto S^2_{\text{local}}$, in agreement with collective lipid dynamics (5, 6). This square-law dependence is a direct manifestation of Fermi's golden rule (4). Furthermore, by describing collective order-director fluctuations with a single elastic constant approximation, a direct connection to the Helfrich bending energy can be made (7). These concepts have been clearly validated in NMR studies of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) membranes containing cholesterol (6, 7). A model-free comparison between DMPC-cholesterol data and the DOPC-cholesterol series in ref. 1 shows the same trends in spectral relaxations associated with increased bending rigidity caused by cholesterol.

Correspondingly, in NSE the dynamics are measured as motional correlations embedded in the intermediate scattering function $I(q,t)$, the Fourier transform of the van Hove correlation function. The motional correlations as a function of Fourier time t allow for the separation of relaxation timescales from material properties by $I(q,t)/I(q,0) = \exp[-(\Gamma t)^{2/3}]$, where Γ is the time-independent (but q-dependent) relaxation rate. For bending fluctuations, the relaxation rates are directly linked to the Helfrich bending elasticity through the Zilman–Granek theory (3), contrary to assertions by Nagle et al. (2). Model-free observations of slower NSE decays, as detected for DOPC-cholesterol membranes, are typically associated with bilayer stiffening. While alternative interpretations are possible, our conclusions are based on well-accepted NSE data analysis methods (8, 9). Importantly, our results are consistent with recent NSE observations showing that elastic membrane properties, including bending rigidity κ, scale with molecular packing (10). We also note that the use of a modified polymer brush model—with redefined mechanical thickness—to relate κ to the area compressibility modulus has been validated by MD simulations of

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saturated and unsaturated membranes containing cholesterol (11), a point ignored in ref. 2. From our independent NSE, NMR, and MD simulation results, a universal stiffening effect of cholesterol on lipid membranes is inferred.

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