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With Lipid Rafts, Context Is Everything

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The lipid makeup of the mammalian plasma membrane (PM) continues to hold the fascination of biophysicists. Although it has long been known that the PM contains hundreds of chemically distinct lipids and a great deal of cholesterol (40% or more by mole fraction), we are still piecing together the puzzle of why cells evolved this compositional complexity. A major step forward was the realization that nonrandom mixing of different lipids could impart functionality, such as the sorting of proteins based on a differential affinity for membrane environments of different composition and physical properties. This crucial observation prompted the lipid raft hypothesis, which singled out a particular preferential interaction—between sphingolipids and cholesterol—as the key driving force for the formation of functional membrane domains (1).

Further insight came with the discovery that model membranes mimicking the PM composition can spontaneously demix into coexisting fluid phases that differ in chain order and other properties. A useful minimal system for studying fluid-fluid phase separation in bilayers is cholesterol and two lipids with a substantial difference in their gel-to-fluid transition temperatures, often referred to as low-melting and high-melting (or low-Tm and high-Tm (melting temperature)) lipids (2). In the absence of cholesterol, immiscibility of the low-Tm and high-Tm lipids drives a gel-fluid phase separation. Cholesterol, when added to the mixture, modifies the properties of both phases but has an especially remarkable effect on the solid gel: past a threshold concentration, cholesterol abolishes long-range crystalline order while preserving the mostly all-*trans* configuration of the lipid chains, resulting in a state of matter that has some properties of a fluid (e.g., fast lateral diffusion) and some properties of a solid (e.g., high chain orientational order). This liquid-ordered (Lo) phase of model membranes, enriched in cholesterol and high-Tm lipid, shares many characteristics with raft domains in cells and has justifiably received considerable experimental attention. Yet, with the focus of the raft hypothesis on cholesterol- and sphingolipid-rich domains, it is easy to overlook that, like yin and yang, the behavior of the raft is inextricably linked to that of the more disordered sea surrounding it. It is this important point that the study by Nyholm et al. (3) addresses in the current issue of *Biophysical Journal*.

Nyholm et al. (3) choose as their primary variable the low-Tm lipid that comprises the liquid-disordered (Ld) fluid phase. Here, some historical context is needed: in studies of ternary mixtures, two low-Tm lipids—POPC and DOPC—stand out for the frequency with which they are used. The choice of POPC is an obvious one from the standpoint of the PM lipidome because its saturated 16:0 chain and unsaturated 18:1 chain represent the average hydrocarbon composition of a low-Tm phospholipid. The motivation for DOPC, with its two 18:1 chains, is less clear because lipids with unsaturations in both chains are not common in the PM. Rather, its popularity may be due to the ease with which, in mixtures, it forms micron-sized liquid domains that are readily visible with fluorescence microscopy. Nyholm et al. (3) use both of these lipids in their study in addition to dimonounsaturated lipids with shorter (14:1-PC) or longer (20:1-PC) chains. Their goal is to determine how chain length mismatch between the low-Tm lipid and high-Tm lipid (here, palmitoyl sphingomyelin (PSM)) influences the properties and formation of Lo domains.

Using deuterium NMR, Nyholm et al. (3) first show that the low-Tm lipid can strongly alter PSM dynamics. When 20 mol % PSM was added to different low-Tm bilayers at room temperature (Fig. 2), the authors found that PSM became progressively more disordered as the low-Tm chain length decreased (at physiological temperature, the differences between low-Tm lipids with the longest chains were much smaller, although the 14:1-PC matrix resulted in considerably more disorder along the entire length of the PSM N-acyl chain). Each of these binary mixtures with 20 mol % PSM resulted in a uniform Ld phase, which the authors demonstrated using the

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fluorescence of *trans*-parinaric acid as a reporter for the onset of gel domains. Indeed, they showed that each of the studied low-Tm bilayers could accommodate a similar amount of PSM (between 27 and 33 mol %) before a PSM-rich gel phase separated from the Ld matrix (Fig. 5, *upper*).

Nyholm et al. (3) then turned their attention to the effect of cholesterol. Using the same *trans*-parinaric acid assay, but titrating PSM into low-Tm bilayers containing 20 mol % cholesterol, they found a dramatic difference in the amount of PSM required to trigger the formation of the Lo phase: PSM solubility in the cholesterol-containing Ld phase decreased more than threefold in the low-Tm series POPC > 20:1-PC > 18:1-PC > 14:1-PC (Fig. 5 *upper*), in stark contrast to the much smaller differences in solubility found in the absence of cholesterol. Clearly, cholesterol enhances the immiscibility of low-Tm lipid with PSM (and thereby facilitates Lo domain formation) in a manner that depends on the length of the unsaturated chains. The authors also looked at the affinity of a fluorescent cholesterol reporter (cholestatrienol) for the different low-Tm bilayers, finding that cholestatrienol has increased affinity when 1) the low-Tm lipid has longer rather than shorter unsaturated chains, 2) the low-Tm lipid has one saturated and one unsaturated chain rather than two unsaturated chains (i.e., POPC compared to DOPC), and 3) the bilayer also contains 20 mol % PSM (Fig. 3). Combining these observations reveals a strong correlation between cholesterol's relative affinity for PSM and a given low-Tm lipid, and the change in PSM solubility in cholesterol-containing bilayers composed of that same low-Tm lipid (Fig. 5, *lower*). In other words, cholesterol—which interacts strongly with PSM, less so with POPC, and increasingly less so with unsaturated lipids of shorter chain lengths—does not much change the solubility of PSM in a POPC bilayer, but dramatically lowers the solubility of PSM in a 14:1-PC bilayer. The biological implication is that a cell can potentially tune the threshold for raft formation by adjusting the average length of its unsaturated low-Tm lipids.

Finally, Nyholm et al. (3) examined the properties of the coexisting fluid phases using a clever NMR experiment that allowed them to separately quantify PSM order in the Ld and Lo environments. In line with their other observations, they found that PSM in the Ld matrix became progressively more disordered as the chain length of the low-Tm lipid decreased (Fig. 6, *lower*). In contrast, PSM in the Lo phase had a similar order regardless of the low-Tm lipid (Fig. 6, *upper*), presumably because of the relatively small amount of disordered lipid that can be accommodated in the Lo phase. NMR also revealed that domains persisted to higher temperatures for shorter low-Tm lipids, a result that was verified using Förster resonance energy transfer and differential scanning calorimetry.

The study by Nyholm et el. (3) continues a line of research aimed at demystifying PM complexity by building a comprehensive set of rules for lipid/cholesterol interactions, which may then be used to predict membrane properties. Previous studies from some of the same authors examined the role of lipid headgroup (4) and the extent of chain unsaturation in mixed-chain low-Tm lipids (5). The present study adds a new variable: changing the chain length of the low-Tm lipid while keeping the total number of double bonds fixed at one per chain. An earlier report had concluded that the extent of unsaturation, rather than the length of the hydrocarbon chains, played a dominant role in determining miscibility with PSM in the presence of cholesterol (5). Nyholm et al. (3) clarifies that in fact, chain length plays at least an equally important role. It is known that cholesterol is forced into unfavorable orientations in thin bilayers (6), so perhaps these observations can be combined into a more general rule that the thickness of the disordered phase—which depends on both the length and degree of unsaturation of the low-Tm lipid—relative to the ordered phase governs their mutual interaction with cholesterol. (Missing from the experimental approach of Nyholm et al. (3) is a direct measurement of bilayer thickness, which could be obtained for example from small-angle scattering.) Alternatively, perhaps the relative order of the coexisting phases, which in addition to the deuterium NMR used by Nyholm et al. (3) can be measured with fluorescent probes like laurdan, is a unifying variable. A complicating factor is that these properties are likely to be highly correlated, making the task of determining which (if any) is the underlying driver a difficult one.

In what new directions could this fruitful line of experiments go? Advances in lipidomics are revealing surprising new details of PM composition including an abundance of plasmalogen species (7), yet very little is known about the influence of these unusual lipids on membrane structure or mixing behavior. It may also be informative to correlate the now extensive library of cholesterol partitioning data with other physical properties of domains, such as quantitative measurements of their size. A wealth of data (including observations in Nyholm et al. (3)) supports the conclusion that one of the most biologically abundant chain motifs, 1-palmitoyl-2-oleoyl, robustly produces nanoscopic domains in ternary model membranes (8) suggesting that lipids like POPC may be key players in a cell's ability to control raft size. These mixed-chain lipids may set a baseline condition in which small clusters of ordered lipids and cholesterol can be nudged to coalesce into larger platforms by local changes in lipid composition (e.g., by delivery of polyunsaturated or shorter-chain low-Tm species to the PM) and thereby induce changes in the spatial organization of membrane-resident proteins.

One issue not addressed by Nyholm et al. (3) is that of PM compositional asymmetry. The rules that govern lipid

and cholesterol interactions in symmetric bilayers can tell only part of the story. Theory predicts that interactions occurring at the midplane of asymmetric bilayers can couple to in-plane interactions and change the lateral organization of lipids in both leaflets (9). It remains to be seen whether continued improvements in the preparation of asymmetric model membranes (10) can be combined with an approach like that of Nyholm et al. (3) to finally solve the riddle of the PM lipidome.

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