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The Structural Role of Cholesterol in Cell Membranes: From Condensed Bilayers to Lipid Rafts

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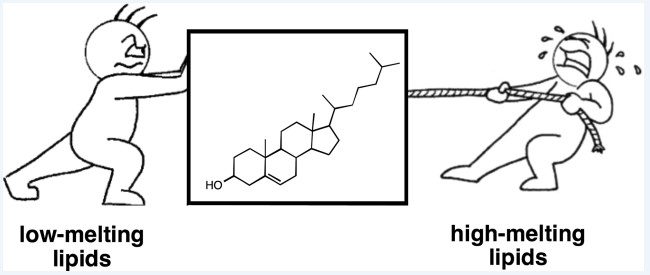
CONSPECTUS: Defining the two-dimensional structure of cell membranes represents one of the most daunting challenges currently facing chemists, biochemists, and biophysicists. In particular, the time-averaged lateral organization of the lipids and proteins that make up these natural enclosures has yet to be established. As the classic Singer−Nicolson model of cell membranes has evolved over the past 40 years, special attention has focused on the structural role played by cholesterol, a key component that represents ca. 30% of the total lipids that are present. Despite extensive studies with model membranes, two fundamental issues have remained a mystery: (i) the mechanism by which cholesterol condenses low-melting lipids by uncoiling their acyl chains and (ii) the thermodynamics of the interaction between cholesterol and high- and low-melting lipids. The latter bears directly on one of the most popular notions in modern cell biology, that is, the lipid raft hypothesis, whereby cholesterol is thought to combine with high-melting lipids to form "lipid rafts" that float in a "sea" of low-melting lipids.



In this Account, we first describe a chemical approach that we have developed in our laboratories that has allowed us to quantify the interactions between exchangeable mimics of cholesterol and low- and high-melting lipids in model membranes. In essence, this "nearest-neighbor recognition" (NNR) method involves the synthesis of dimeric forms of these lipids that contain a disulfide moiety as a linker. By means of thiolate−disulfide interchange reactions, equilibrium mixtures of dimers are then formed. These exchange reactions are initiated either by adding dithiothreitol to a liposomal dispersion to generate a small amount of thiol monomer or by including a small amount of thiol monomer in the liposomes at pH 5.0 and then raising the pH to 7.4. We then show how such NNR measurements have allowed us to distinguish between two very different mechanisms that have been proposed for cholesterol's condensing effect: (i) an umbrella mechanism in which the acyl chains and cholesterol become more tightly packed as cholesterol content increases because they share limited space under phospholipid headgroups and (ii) a template mechanism whereby cholesterol functions as a planar hydrophobic template at the membrane surface, thereby maximizing hydrophobic interactions and the hydrophobic effect. Specifically, our NNR experiments rule out the umbrella mechanism and provide strong support for the template mechanism.

Similar NNR measurements have also allowed us to address the question of whether the interactions between low-melting kinked phospholipids and cholesterol can play a significant role in the formation of lipid rafts. Specifically, these NNR measurements have led to our discovery of a new physical principle in the lipids and membranes area that must be operating in biological membranes, that is, a "push−pull" mechanism, whereby cholesterol is pushed away from low-melting phospholipids and pulled toward high-melting lipids. Thus, to the extent that lipid rafts play a role in the functioning of cell membranes, low-melting phospholipids must be active participants.

1. INTRODUCTION

The classic Singer−Nicolson model has served as a useful starting point for defining the structure of cell membranes for over 40 years.[1] According to this model, phospholipid bilayers contain modest amounts of integral proteins that are strongly bound and largely confined to the hydrocarbon interior of the membrane (Figure 1). In addition, a modest amount of peripheral proteins are present, which are weakly bound to the membrane's inner and outer surface. This model also assumes that the proteins as well as the lipids are randomly arranged and that they undergo rapid lateral diffusion. Transbilayer movement (i.e., "flip-flop") is considered to be unlikely because the polar headgroups would be required to enter the nonpolar membrane interior. Finally, cell membranes are viewed as being asymmetric due to inner and outer leaflets that differ in composition.

Based on a large number of biochemical and biophysical studies that have been reported since its introduction, the Singer−Nicolson model has continued to evolve.[2−5] A more modern version now includes (i) a high density of proteins associated with the lipid framework, (ii) protein−membrane interactions ranging from very strong to very weak, (iii) lateral heterogeneity existing among the lipids and proteins, (iv) lipids capable of undergoing transbilayer motion, (v) many integral proteins having only a small segment actually inserted into the

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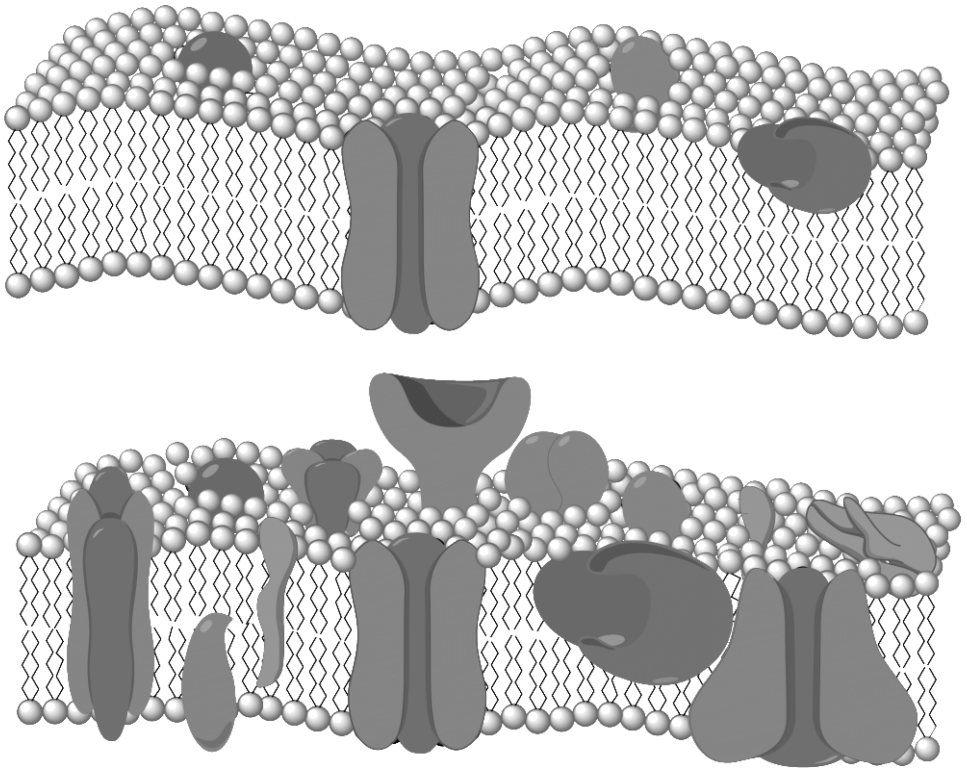

Figure 1. Stylized illustrations of the classic Singer–Nicolson model of the structure of cell membranes (top) and a more modern version showing a higher density of proteins (bottom).

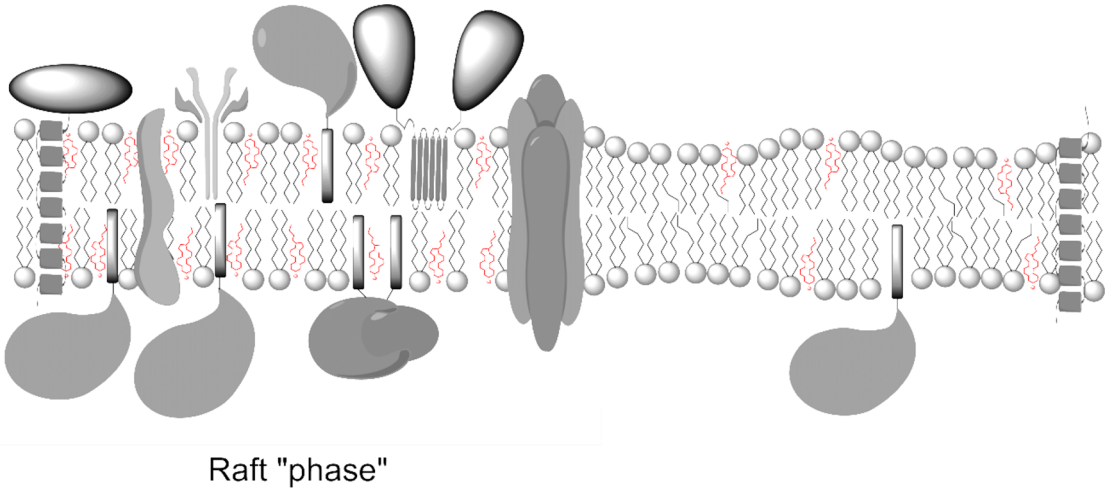

Figure 2. Stylized illustration of a raft "phase" (left half) coexisting with a nonraft region (right half).

bilayer, (vi) membrane proteins interacting with other membrane proteins, (vii) bilayer thicknesses that are constantly fluctuating, and (viii) some of the integral proteins strongly favoring certain lipids over others as "boundary lipids".[4,5]

One aspect of cell membranes that has received special interest in recent years has focused on the "lipid raft hypothesis".[6] Thus, based on membrane fragments that were found to be resistant to disruption by Triton X-100 at 4 °C (i.e., detergent-resistant membrane (DRM) fragments), it was initially hypothesized that such fragments correspond to discrete domains within cell membranes in their native state (Figure 2).[7,8] The fact that these DRM fragments were rich in cholesterol and high-melting sphingolipids (i.e., sphingolipids having a gel to liquid-crystalline phase transition temperature in excess of 37 °C) also led to the belief that these "lipid rafts" float in a sea of the membrane that is rich in low-melting phospholipids (having T_m < 37 °C) and poor in cholesterol. However, subsequent studies of DRM fragments and model systems have led some researchers to question the correctness of these interpretations and the usefulness of these fragments for investigating membrane organization.[9−15] Specifically, the fact that their composition was found to be strongly dependent on the nature of the nonionic detergent used, the concentration of the detergent, and the time that the membranes are exposed to detergent has raised doubts about their significance.[13−15] It has also been argued that the physical state of cell membranes is likely to change on going from 37 to 4 °C and that DRM fragments are unlikely to reflect membrane organization at 37 °C.

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More modern methods that have been applied to study lipid domains have included single particle tracking (SPT), fluorescence correlation spectroscopy (FCS), fluorescence resonance energy transfer (FRET), stimulated emission depletion nanoscopy (STED), and photoactivated localization microscopy (PALM).[6,16−18] In most instances, an observed clustering of specific proteins has been used as inferential evidence to support the existence of lipid rafts. However, the extent to which such clustering is driven by protein−protein interactions remains uncertain.[19]

In the absence of cholesterol, pure phospholipids and sphingolipids form well-defined bilayers. When maintained below their gel to liquid-crystalline phase transition temperature, T_m, these bilayers exist in a gel (or solid-like) state, in which their hydrocarbon chains are fully extended in an all-anti conformation (Figure 3).[15] At temperatures in excess of T_m, gauche conformers

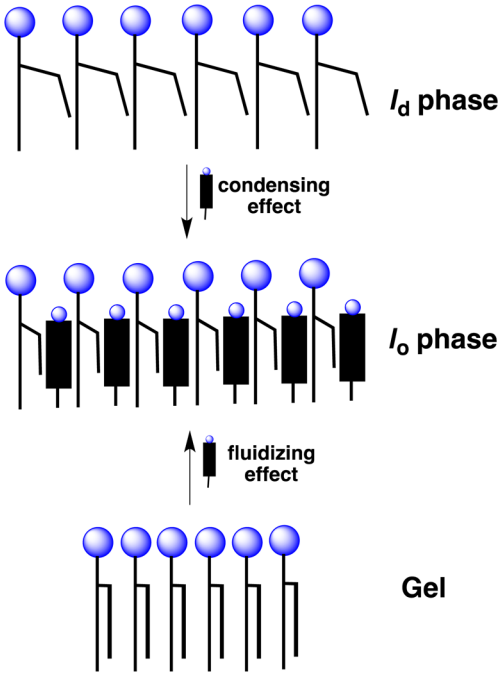

Figure 3. Stylized illustrations showing the liquid-disordered (l_d), liquid-ordered (l_o), and gel phases.

are introduced into the acyl chains and the bilayer undergoes a thinning. The resulting liquid-crystalline phase (also referred to as the liquid-disordered, l_d, phase) is characterized by a higher degree of fluidity with the lipids having greater lateral mobility. When added to high-melting lipids, cholesterol exerts a *fluidizing effect* that leads to the introduction of gauche conformers, a thinning of the bilayer, and lipids having greater lateral mobility.[20,21] When a sufficient amount of cholesterol has been added, the membrane is fully converted into a new phase that has been termed the liquid-ordered, l_o, phase.[15,22,23] In contrast, cholesterol exerts a *condensing effect* on the liquid-disordered phase. Here, the sterol reduces the number of gauche conformers, increases the thickness of the bilayer, and reduces the lateral mobility of the lipids.[15] Thus, cholesterol tends to move both the gel and l_d phases toward a common l_o phase having an intermediate thickness, an intermediate number of gauche conformers, and an intermediate degree of compactness, fluidity, and lateral mobility.[15]

The fact that mammalian cell membranes are rich in cholesterol, corresponding to ca. 30% of the total lipid content, suggests that most if not all of the high- and low-melting lipids that are present exist in an intermediate phase (or phases) that resembles the l_o phase. How many domains (e.g., "lipid rafts") exist within cell membranes, as well as their average size, composition, and lifetimes, remains to be established. It is noteworthy, in this regard, that recent studies of model membranes have lent support for the possible existence of lipid rafts by demonstrating the feasibility of forming domains of varying size.[24−28]

In an effort to gain a fundamental understanding of domain formation in fluid bilayers, we have taken a bottom-up approach. Specifically, our goal has been to quantify the free energy of interaction between different neighboring lipids in model membranes. Such information can then be used in Monte Carlo simulations to produce physical pictures of thermodynamically favored states at the molecular level.[29] Although lipid−lipid interactions have been investigated by a variety of physical methods such as differential scanning calorimetry, fluorescence energy transfer, isothermal titration calorimetry, analysis of phase diagrams, and cyclodextrin partitioning, we sought a method that would be direct (i.e., would not require any matching of experimental data with theoretical curves) and would be capable of detecting energy differences down to tens of calories per mole.[29,30] As has been shown by Monte Carlo simulations, such small differences in energy are sufficient to produce large changes in lipid domain size distributions.[28,29]

With this goal in mind, we have devised a chemical approach for investigating lipid−lipid interactions that we have termed, nearest-neighbor recognition (NNR). In this Account, we describe how this method works and show how it has provided new insight into two fundamental and important issues relating to the action of cholesterol in lipid bilayers, that is, its condensing action and its interaction with low-melting kinked phospholipids. The relevance of the latter to the formation of putative lipid rafts is also discussed.

2. THE NEAREST-NEIGHBOR RECOGNITION METHOD

2.1. How It Works

A summary of how the nearest-neighbor recognition method works is illustrated in Figure 4. In essence, the NNR technique involves dimer interchange reactions that afford equilibrium compositions, which reflect the net interaction between two different membrane components. To date, all NNR experiments have been carried out using thiolate−disulfide interchange reactions. Such reactions are particularly attractive since they can be readily "turned on" or "turned off" by simple pH adjustment; that is, they proceed at moderate rates at pH 7.4 but can be essentially stopped at pH 5.0.[31,32] In a typical NNR experiment, two components of interest (**A** and **B**) are converted into exchangeable dimers (i.e., homodimers **AA** and **BB** and also a heterodimer **AB**) using a suitable disulfide linkage. These dimers are then included in a host membrane that is maintained in a given phase (e.g., the liquid-disordered or liquid-ordered state), and the monomers are then allowed to undergo interchange via thiolate−disulfide exchange.[33] These reactions are initiated either by adding *threo*-dithiothreitol to a liposomal dispersion to generate a small amount of thiol monomer or by including a small amount of thiol monomer in the liposomes at pH 5.0 and then raising the pH.[34] To ensure that the pH of the aqueous interior of the liposomes rapidly equilibrates with the external aqueous phase, a small amount of an ionophore (monensin) is also included in the dispersion. Aliquots are then quenched

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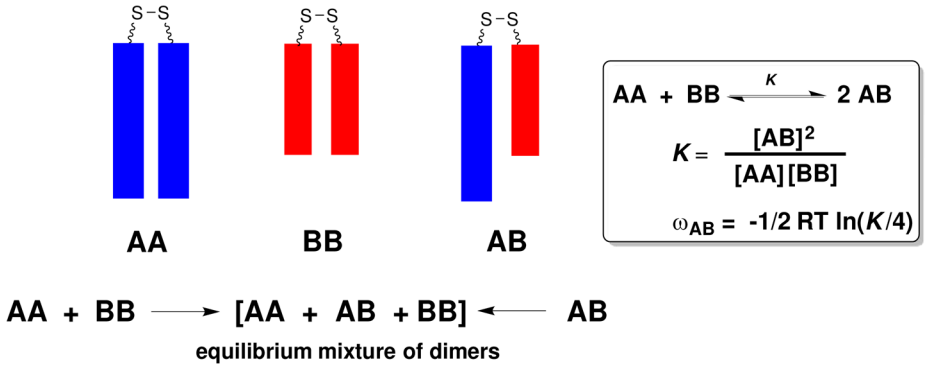
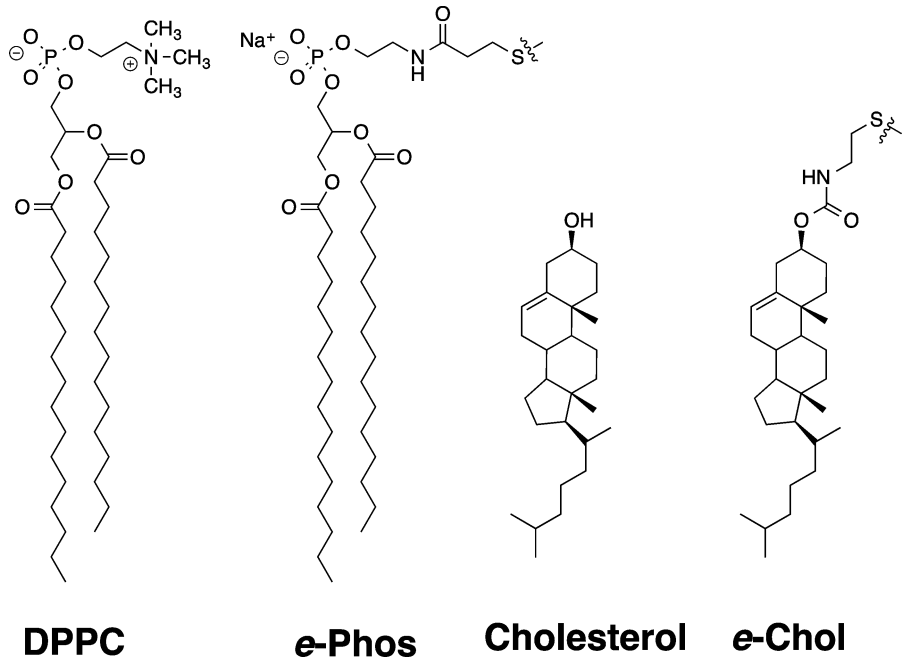


Figure 4. Basis of the nearest-neighbor recognition (NNR) method.

Chart 1



as a function of time by lowering the pH to 5.0 and freezing the dispersion (−20 °C). Analysis of the dimers is then made by HPLC after the samples have been thawed to room temperature and extracted with $CHCl_3/CH_3OH$ (2/1, v/v) and the residual thiols have been "capped" using 2,2′-dipyridyldisulfide.

Equilibrium mixtures are then used to calculate the equilibrium constant, K, where $K = [\mathbf{AB}]^2/([\mathbf{AA}][\mathbf{BB}])$. When lipid monomers **A** and **B** mix ideally, this is reflected by an equilibrium constant that equals 4.0. Favored homoassociations are indicated by values of K that are less than 4.0, while favored heteroassociations are indicated when $K > 4.0$. Taking statistical considerations into account, it can be shown that the nearest-neighbor interaction free energy between **A** and **B**, ω_{AB}, is then given by $\omega_{AB} = -{}^1/_2 RT \ln(K/4)$.[29]

Although the nearest-neighbor recognition method was originally designed for lipid–lipid interactions, we have recently extended it to the study of lipid–lipidated peptide interactions.[35] Currently, we are applying it to the study of lipid–peptide and lipid–protein interactions. Other researchers have used a similar approach for studying the thermodynamics of protein–protein interactions in lipid bilayers.[36]

2.2. An Exchangeable Phospholipid and an Exchangeable Sterol

Two lipids of which we have made extensive use in our NNR studies are the exchangeable phospholipid, ***e*-Phos**, and the exchangeable sterol, ***e*-Chol**, which are shown in Chart 1. The synthetic methods used to prepare the corresponding homodimers, {***e*-Phos–*e*-Phos**} and {***e*-Chol–*e*-Chol**}, and heterodimer, {***e*-Phos–*e*-Chol**} are shown in Scheme 1.[37−39]

Our design of ***e*-Phos** was based on the assumption that it would have nearest-neighbor interactions that are very similar to those of DPPC with other lipids. Similarly, ***e*-Chol** was expected to have nearest-neighbor interactions that are very similar to those of cholesterol. In the following sections, we summarize the mixing behavior of ***e*-Phos** with ***e*-Chol** in the l_d and l_o states and provide several lines of evidence that strongly support these assumptions.

2.3. Nearest-Neighbor Interactions between *e*-Phos and *e*-Chol in the Liquid-Disordered and Liquid-Ordered States

Because the phase properties of membranes made from DPPC and cholesterol have been well-defined, we have used them as host membranes for investigating the nearest-neighbor interactions between ***e*-Phos** and ***e*-Chol** in the l_d and l_o states.[40]

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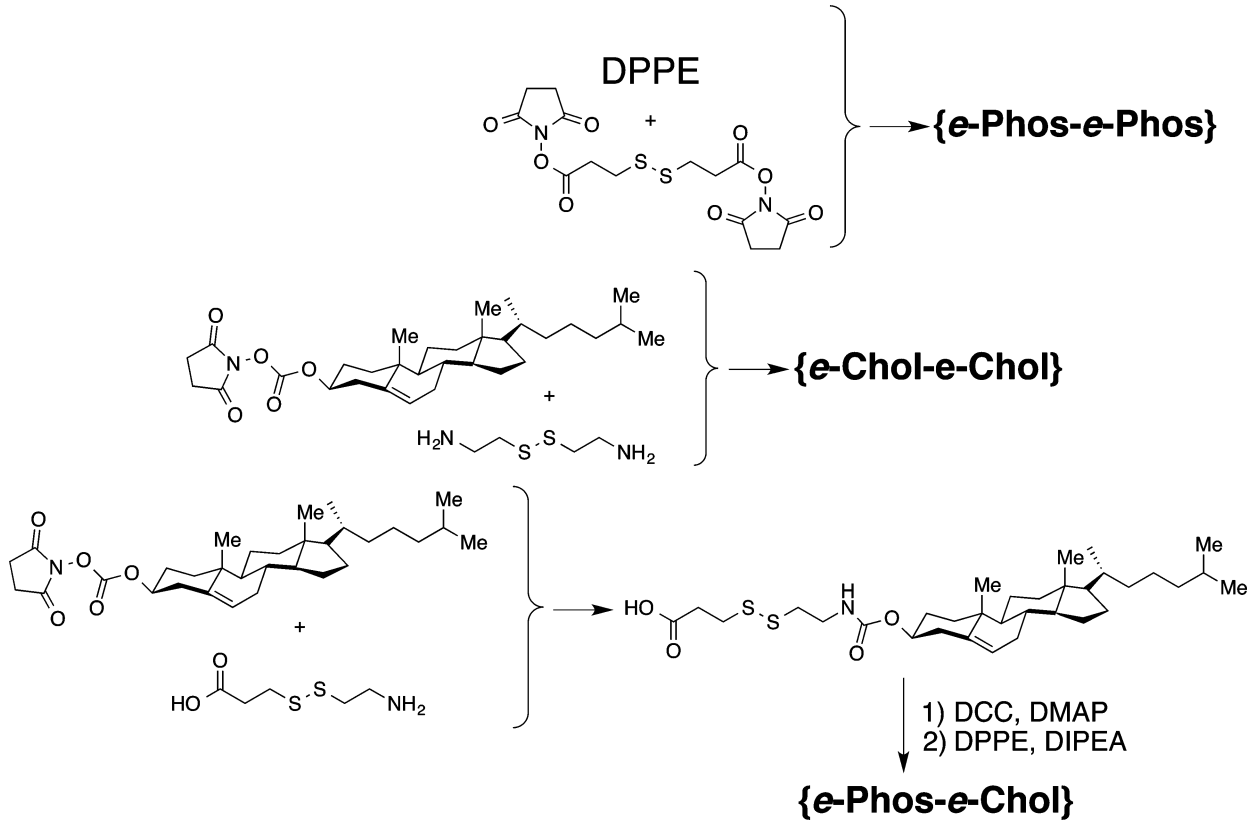
Scheme 1



At temperatures above its T_m value of 41 °C, DPPC membranes are fully in the l_d state. From 41 to ca. 70 °C, as increasing amounts of cholesterol are added, the membrane is converted to a liquid-disordered/liquid-ordered (l_d/l_o) coexistence region according to its phase diagram. When 40 mol % of cholesterol is present, the membrane is fully converted into the l_o phase even at temperatures that are well below physiologically relevant 37 °C. Using host membranes made from DPPC and 2.5 mol % cholesterol (in the form of ***e*-Chol**), we have found that ***e*-Chol** mixes randomly with ***e*-Phos** at 45 °C; that is, $K = 3.9 \pm 0.3$, which corresponds to $\omega_{AB} = 12 \pm 19$ cal/mol.[32] In contrast, when the level of cholesterol was increased to 40 mol % to fully convert the membrane to the l_o phase, there is a strong preference for ***e*-Chol** and ***e*-Phos** to become nearest-neighbors; here $K = 9.2 \pm 0.2$ and $\omega_{AB} = -260 \pm 6.3$ cal/mol of phospholipid.[32] Although other studies have demonstrated that high-melting lipids such as DPPC favor association with cholesterol in monolayers assembled at the air/water interface, these NNR measurements allow one to quantify directly the free energy of interaction for these exchangeable mimics in the physiologically relevant fluid bilayer state.[41]

2.4. *e*-Phos and *e*-Chol Are Excellent Mimics of DPPC and Cholesterol

Despite the introduction of a disulfide moiety, a difference in headgroup composition, and a net negative charge, ***e*-Phos** has proven to be an excellent mimic of DPPC. The fact that ***e*-Phos** possesses this negative charge also makes it an excellent mimic of the corresponding phosphatidylglycerol, that is, DPPG. Similarly, ***e*-Chol** has proven to be an excellent mimic of cholesterol. Several lines of evidence strongly support these claims. First, DPPC and ***e*-Phos**, having identical acyl chains, show nearly identical melting behavior.[42] Thus, DPPC is characterized by a T_m of 41.5 °C and an enthalpy (ΔH) of 8.7 kcal/mol of phospholipid. The corresponding values for ***e*-Phos** are 41.9 °C and 9.3 kcal/mol of phospholipid. This is exactly analogous to the situation for zwitterionic phosphatidylcholines and negatively charged phosphatidylglycerols, where identical acyl chains lead to nearly identical melting behavior and ideal mixing.[43] Second, as one would expect, ***e*-Phos** exhibits ideal mixing with an exchangeable zwitterionic analog, which was also synthesized in our laboratory, in cholesterol-rich and cholesterol poor membranes.[44] In addition, ***e*-Phos** and this exchangeable zwitterionic analog show nearly identical melting behavior and monolayer properties.[44,45] Because the synthesis of this exchangeable zwitterionic analog is considerably more involved than that of ***e*-Phos**, we have made extensive use of the latter in most of our NNR investigations. Third, ***e*-Chol** exhibits monolayer properties and condensing behavior that are nearly identical to that of cholesterol.[39] Fourth, the interaction between ***e*-Phos** and ***e*-Chol** in host membranes made from DPPC and cholesterol is indistinguishable from that found in liposomal membranes made exclusively from these exchangeable mimics.[46] In addition, the temperature dependence of the nearest-neighbor interactions is also indistinguishable. Taken together, these findings indicate that ***e*-Phos** and ***e*-Chol** have the same average microenvironment in both membranes and that these membranes contain similar proportions of the liquid-disordered and liquid-ordered phase at a given temperature. Fifth, the conversion from the l_d to the l_o phase, which is apparent from NNR measurements in membranes made exclusively from ***e*-Phos** and ***e*-Chol**, occurs exactly over the same range of sterol concentrations as that found in membranes made from DPPC and cholesterol, as established by a variety of methods.[39,40] This finding provides further evidence that the interactions between ***e*-Phos** and ***e*-Chol** are very similar to those between DPPC and cholesterol. Finally, the fact that these NNR

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measurements can detect very slight changes in membrane compactness (e.g., on going from a fully l_d to a fully l_o state) also demonstrates their usefulness as a tool for quantifying the condensing power of sterols with high precision.[47]

3. CHOLESTEROL'S CONDENSING EFFECT

3.1. An Umbrella versus a Template Mechanism

The ability of cholesterol to condense fluid-phase phospholipids has been known for almost a century.[48] Despite numerous investigations, the mechanism by which this sterol uncoils the acyl chains of phospholipids has remained a mystery. Two distinct mechanisms that have been proposed to account for cholesterol's condensing effect are (i) an umbrella mechanism and (ii) a template mechanism (Figure 5).[39,49]

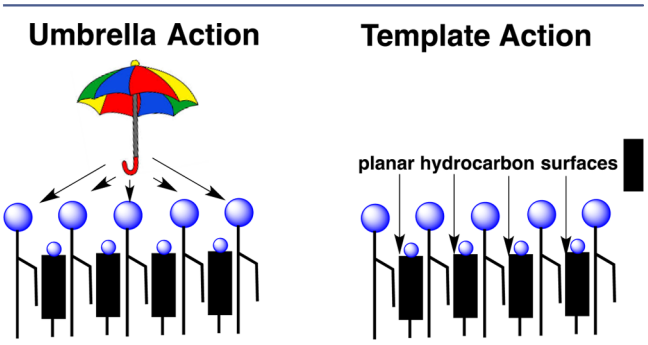


Figure 5. Stylized illustrations of the umbrella and template mechanisms of cholesterol's condensing effect.

According to the umbrella mechanism, "acyl chains and cholesterols become more tightly packed as cholesterol content increases, because they share the limited space under phospholipid headgroups. The hydrophobic nature of cholesterol thus forces cholesterol and acyl chains together."[49] In other words, the headgroups act like umbrellas in keeping the acyl chains of the phospholipid and the sterol nucleus dry. In contrast, the template mechanism assumes that hydrophobic interactions are maximized when the flexible acyl chains complement, perfectly, the planar sterol nucleus, which results in tight packing of the membrane.

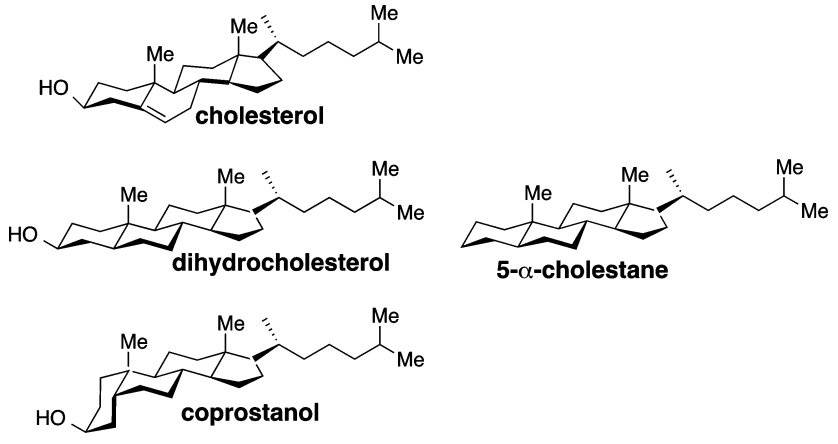
3.2. Distinguishing between an Umbrella and a Template Mechanism

To distinguish between an umbrella and a template mechanism, we have compared the condensing power of cholesterol, dihydrocholesterol, and coprostanol (Chart 2).

The basis for this comparison is illustrated in Figure 6 and is briefly summarized by the following: Because cholesterol and dihydrocholesterol have a similar "flat" structure, they have, essentially, the same cross-sectional area.[50] Since they both occupy the same area and are flat and since they are both capable of producing a high number of close hydrophobic contacts with neighboring acyl chains, both sterols are expected to have a similar condensing power according to both the umbrella and the template mechanisms. However, in the case of coprostanol, which has a significantly larger cross-sectional area due to the *cis*-fusion of its A and B rings, the umbrella and template mechanisms lead to predictions that are diametrically opposed to one another.[50] Specifically, if the umbrella mechanism were operating, coprostanol is expected to have a stronger condensing effect than cholesterol and dihydrocholesterol since crowding (condensation) would be enhanced beneath the phospholipid's headgroup to keep the acyl chains and the sterol nucleus as dry as possible. If a template mechanism were operating, coprostanol is expected to have a weaker condensing effect since fewer close hydrophobic contacts with neighboring acyl chains are possible due to the sterol's curvature. In principle, therefore, a quantitative comparison of the condensing power of these three sterols should allow one to distinguish between an umbrella and a template mechanism.

Nearest-neighbor recognition experiments have been carried out using ***e*-Chol** and ***e*-Phos** (2.5 mol % of each monomer) in a "sensing mode" to measure changes in the compactness of host membranes made from DPPC and each of these sterols. When high levels of cholesterol (ca. 40 mol %) were replaced with coprostanol, a dramatic decrease in condensing action was observed; that is, K was reduced from 9.6 ± 0.6 to 6.3 ± 0.3.[51] In contrast, similar replacement with dihydrocholesterol showed a condensing effect that was indistinguishable from that of cholesterol.[47,51] These findings, along with results obtained from monolayer studies at the air/water interface and fluorescence measurements of general polarization for Laurdan (a phase-sensitive probe), clearly demonstrated that cholesterol and dihydrocholesterol have a similarly strong condensing power while coprostanol's is much weaker. Thus, these results provide strong support for a template mechanism of condensation and a strong argument against an umbrella mechanism.

Chart 2



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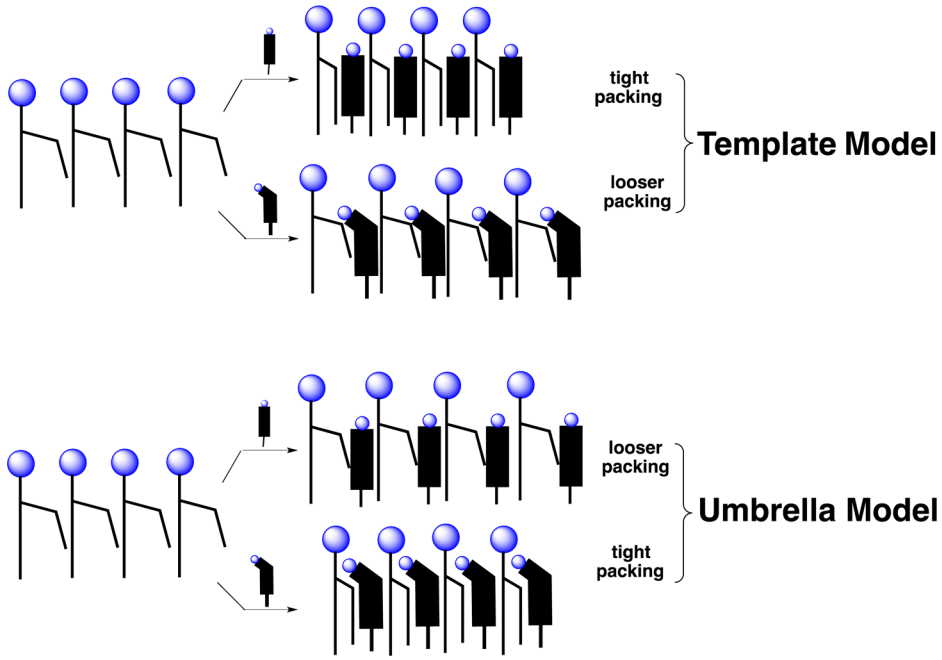

Figure 6. Stylized illustrations of the condensing action by flat and bent sterols interacting with a segment of a phospholipid bilayer according to template and umbrella mechanisms.

To gain further insight into this condensing effect, we have also compared the condensing power of dihydrocholesterol with that of 5-α-cholestane, a sterol analog that is devoid of a C-3 hydroxyl group.[37] In this case, we found that complete replacement of dihydrocholesterol with 5-α-cholestane led to a dramatic decrease in condensing power, as evidenced by $K = 5.2 \pm 0.2$; that is, the condensing effect of this totally hydrophobic steroid was very small. This finding was also confirmed, qualitatively, by Laurdan measurements. Since cholesterol and dihydrocholesterol are anchored to the surface of the bilayer through their hydroxyl group and 5α-cholestane must be confined to the hydrocarbon interior of the membrane (the average location and orientation of 5α-cholestane within the bilayer remain to be established), surface occupancy must be an important factor for cholesterol's condensing effect.

If one adds surface occupancy to the template model, then a simple picture emerges for cholesterol's condensing action. In the liquid-disordered state, the phospholipid's headgroup occupies only about half of its total surface area; the remaining area is occupied by exposed and partially hydrated CH_2 groups. By occupying space at the membrane surface, the sterol replaces these wet CH_2 groups with its hydroxyl group, allowing the acyl chains to undergo partial dehydration and straightening to allow for maximum hydrophobic contact and tight packing. Thus, surface occupancy and the template effect work together to maximize the hydrophobic effect, that is, the release of water from the membrane (Figure 7).

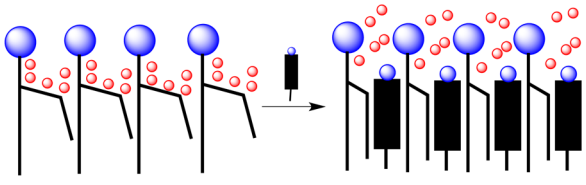

Figure 7. Release of water that accompanies template action by a flat sterol at the membrane surface.

4. THE INTERACTION BETWEEN CHOLESTEROL AND LOW-MELTING "KINKED" PHOSPHOLIPIDS

4.1. Suggestive and Contradictory Results

While the interactions between cholesterol and high-melting lipids have been of considerable interest in recent years due to the lipid raft hypothesis, the interactions between cholesterol and low-melting "kinked" phospholipids have been largely ignored. An important question that one can ask is whether such interactions play a role in the formation of lipid rafts or whether low-melting phospholipids are simply "innocent bystanders".

Naturally occurring phospholipids are low-melting by virtue of the fact that they are "kinked"; that is, they contain one or more *cis*-double bonds located near the center of the acyl chains at the *sn*-2 position of the phospholipid. For example, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), which is the major unsaturated lipid found in eukaryotic membranes, has a T_m value of −3 °C.[52] Whether the net interaction between POPC and other kinked phospholipids with cholesterol is attractive or repulsive has led to some apparently contradictory results.

Previous fluorescence resonance energy transfer experiments in combination with Monte Carlo simulations, as well as isothermal titration calorimetry measurements, strongly suggest that the interaction between POPC and cholesterol is repulsive in the l_d phase.[53,54] Although the interaction between POPC and cholesterol in the l_o phase has not been investigated, experimentally, rough estimates suggested that in bilayers of POPC containing 30 mol % cholesterol (which is presumed to lie in the l_o phase), the free energy of interaction is close to zero; that is, there is no net attraction or repulsion between them.[29] However, recent simulations of ternary mixtures of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and cholesterol suggest that this doubly kinked DOPC molecule has an affinity for cholesterol in the l_o phase.[55]

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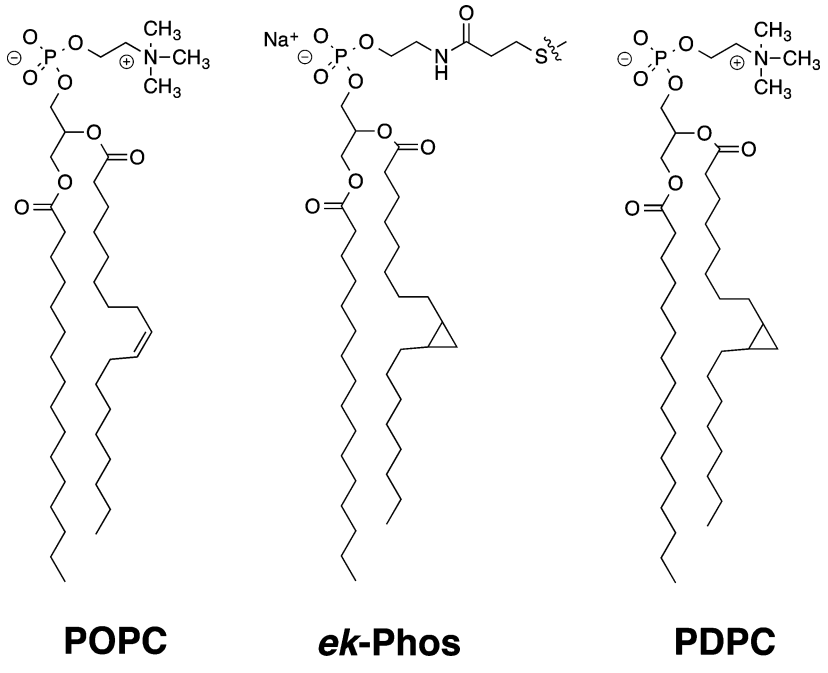
Chart 3



4.2. Nearest-Neighbor Recognition Measurements

To gain insight into the interactions between cholesterol and a kinked phospholipid, we measured ω_{AB} for ***e*-Chol** interacting with an exchangeable and kinked phospholipid, ***ek*-Phos**, in the l_d and l_o phases. Because double bonds are configurationally unstable under NNR conditions, a *cis*-cyclopropyl moiety was used to lock in a "kink" in the acyl of a POPC mimic (Chart 3).[56] As we have previously demonstrated, POPC, ***ek*-Phos**, and 1-palmitoyl-2-dihydrosterculoyl-*sn*-glycero-3-phosphocholine (PDPC) have very similar physical properties; that is, they have nearly identical monolayer behavior and limiting areas of ca. 80 Å^2/phospholipid.[56] In addition, the T_m values for POPC and PDPC are −10 and −3 °C, respectively; the T_m for ***ek*-Phos** has been estimated to be −10 °C.[56] In addition, host membranes made from PDPC show very similar compactness compared with ones made from POPC, as judged by fluorescence measurements using Laurdan as a probe.[57]

Nearest-neighbor recognition measurements that were carried out for ***e*-Chol** and ***ek*-Phos** in host membranes made from PDPC in the l_d phase (45 °C) clearly showed that their net interaction was *repulsive*, that is, ω_{AB} = +160 ± 30 cal/mol of phospholipid.[57] Also, when 40 mol % cholesterol was present, a significant decrease in repulsion was observed, that is, ω_{AB} = +52 ± 38 cal/mol. Similar NNR experiments that were carried out using cholesterol-rich DPPC host membranes in the l_o phase further showed that ***e*-Chol** and ***ek*-Phos** are randomly mixed; that is, ω_{AB} = 0.0 ± 7.9 cal/mol.[57] It is noteworthy that these nearest-neighbor interaction free energies are in excellent agreement with one previously determined from indirect analysis or estimated from differences in interactions between lipid pairs.[29,54] Thus, such agreement adds further confidence to the relevance of these NNR results, since they are fully consistent with lipid−lipid interactions determined by a variety of other methods using natural phospholipids and cholesterol.

4.3. Push−Pull Mechanism for Lipid Raft Formation

The combination of *repulsive* interactions that we have measured for ***e*-Chol** interacting with ***ek*-Phos** in the l_d phase and the *attractive* interactions between ***e*-Chol** and ***e*-Phos** in the l_o phase, affords a simple explanation for the known ability of low-melting, unsaturated phospholipids to stabilize ordered domains generated from high-melting, saturated phospholipids and cholesterol.[58−60] Thus, one now expects that low-melting phospholipids will "push" cholesterol out of disordered regions while high-melting phospholipids will "pull" it into ordered domains (Figure 8). Presumably, the larger the number of kinks that a low-melting phospholipid has, the stronger its "push" and the greater its effectiveness in stabilizing ordered domains. However, this needs to be confirmed by experiment.

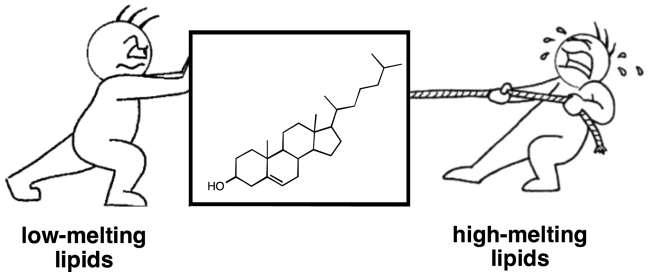


Figure 8. A cartoon illustration of the push−pull mechanism of lipid raft formation.

This new physical principle, the "push−pull" mechanism, whereby cholesterol is pushed away from low-melting phospholipids and pulled toward high-melting lipids, must also be operating in biological membranes *to support the formation of putative lipid rafts through repulsive interactions with cholesterol.*

5. CONCLUSIONS AND PROSPECTS

In this Account, we have shown how a chemical exchange reaction that is carried out in model membranes can provide quantitative insight into the interaction between neighboring lipids in the physiologically relevant fluid bilayer state. We have also shown how such measurements have afforded new insight into the long-known and poorly understood condensing effect that cholesterol has on fluid phospholipids. Related nearest-neighbor

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recognition studies that have been carried out using a low-melting kinked phospholipid have also yielded a new physical principle that must also be operating in biological membranes: *these low-melting phospholipids support the formation of lipid rafts through repulsive interactions with cholesterol*.

We have begun to apply the NNR method to the study of lipid−lipidated peptide interactions, and such studies have already led us to draw an unexpected conclusion, that the sorting of peripheral proteins in cellular membranes via differential lipidation may be more subtle than previously thought.[35] To our great satisfaction, this conclusion was recently supported by fluorescence experiments in live cells.[61] Current efforts in our laboratory are turning toward the use of the NNR method to investigate lipid−peptide and lipid−protein interactions. Given the high density of proteins that exist in cell membranes, we believe that lipid−proteins interactions are likely to play an important role in defining their two-dimensional structure.

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Notes

The authors declare no competing financial interest.

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