Phase Separation in Biological Membranes: Integration of Theory and Experiment

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Key Words

membrane rafts, lipid nanodomains, line tension, continuum theory, Monte Carlo simulation, nanoscopic detection

Abstract

Lipid bilayer model membranes that contain a single lipid species can undergo transitions between ordered and disordered phases, and membranes that contain a mixture of lipid species can undergo phase separations. Studies of these transformations are of interest for what they can tell us about the interaction energies of lipid molecules of different species and conformations. Nanoscopic phases (<200 nm) can provide a model for membrane rafts, specialized membrane domains enriched in cholesterol and sphingomyelin, which are believed to have essential biological functions in cell membranes. Crucial questions are whether lipid nanodomains can exist in stable equilibrium in membranes and what is the distribution of their sizes and lifetimes in membranes of different composition. Theoretical methods have supplied much information on these questions, but better experimental methods are needed to detect and characterize nanodomains under normal membrane conditions. This review summarizes linkages between theoretical and experimental studies of phase separation in lipid bilayer model membranes.

Contents

Nanodomain: a lipid domain in a bilayer membrane with dimensions in the range of 10–200 nm

INTRODUCTION

Lipids and proteins form specialized regions called membrane rafts that are too small to be seen by conventional light microscopy (78). The raft lipids constitute an ordered liquid phase physically distinct—more tightly packed and viscous—from the disordered surrounding lipids. The selective incorporation of specific proteins into rafts may be important for membrane trafficking, signaling, and assembly of specialized structures, e.g., in virus budding, endocytosis, and immune responses (80). For example, clustering of signaling receptor molecules in rafts could substantially promote their ability to interact, enhancing the specificity and efficiency of signal generation from the cell surface (75). Conversely, sequestration of signaling molecules in separate rafts could inhibit signal generation.

The study of rafts has been driven by both theory and experiment, with experimental evidence often lagging behind. In formulating the fluid mosaic model, Singer & Nicolson (96) recognized the existence of heterogeneities at length scales up to 100 nm. In the same year, Shimshick & McConnell (92) proposed that phase separation could facilitate the insertion of proteins into the membrane and enhance membrane trafficking. Within four years, models recognizing the importance of domain structures associated with long-range order were developed (42, 44). However, the first experimental evidence of rafts appeared only in the late 1980s (94, 105). Subsequently, as recent reviews attest (21, 25, 55, 77), interest has grown explosively.

A substantial body of literature now exists that dissects the biophysical interactions governing raft formation and growth as well as lipid phase coexistence in model lipid bilayers (106). However, experimental challenges persist, leaving open fundamental questions about the biophysical properties of rafts. What determines the size and stability of rafts? How do protein molecules within a nanodomain influence its stability and dynamic properties? Underlying many of these challenges are the size, fleeting nature, and complexity of rafts: Rafts vary in lipid and protein composition, with lifetimes determined by interactions among their components (51, 77). Model lipid bilayer systems and novel microscopy techniques are needed to overcome these experimental challenges.

Our objective is to survey the physical principles believed to govern the formation of rafts, theoretical models that embody these principles, and experimental approaches to quantify raft size distributions and dynamics. We focus on model bilayers in which the lipids, cholesterols, and proteins that comprise the system are well defined, but we emphasize that even for these systems much uncertainty persists. Even within this narrow focus, we must omit many important contributions in this brief review. After a brief overview of nanodomains in model bilayers and membrane rafts in living cells, we consider the following:

- What has been learned from theory and computational modeling about whether and under what conditions nanodomains exist and their size distribution and lifetimes? We consider selected examples that illustrate the theoretical methods used and the kinds of information that can be obtained about phase separation, but do not attempt a comprehensive survey.
- What methods are available for detecting and characterizing nanodomains, as well as for validating predictions? What have these methods revealed about the presence of nanodomains in model and biological membranes?
- What are the prospects for the future study of nanodomains in model bilayer membranes and their relationship to membrane rafts?

NANODOMAINS IN MODEL SYSTEMS AND RAFTS IN CELLS

Phase Transitions and Phase Separations in Lipid Bilayer Membranes

Model membranes of defined composition are essential to understanding coexistence of phase domains with different degrees of lipid hydrocarbon chain conformational order (**Figure 1**). Studies of transitions between solid (gel) and liquid (liquid crystalline) phases in membranes were initiated decades ago (39, 64, 92). In single-component systems this transition occurs at a well-defined melting temperature, T_m. Coexistence between solid (S_0) and disordered liquid (L_α) phases is readily observed in two-component mixtures of higher and lower melting lipids. Liquid disordered (L_d or L_α) and liquid ordered (L_o) domains can coexist in binary systems and in ternary mixtures of high- and low-melting lipids and cholesterol. The liquid character of the L_d and L_o phases is confirmed by the relatively rapid lateral and rotational diffusion of lipids in both phases; the lateral diffusion coefficient is about two- to threefold less in the L_o phase (106). Furthermore, these domains deform elastically when exposed to external fluid flow (87).

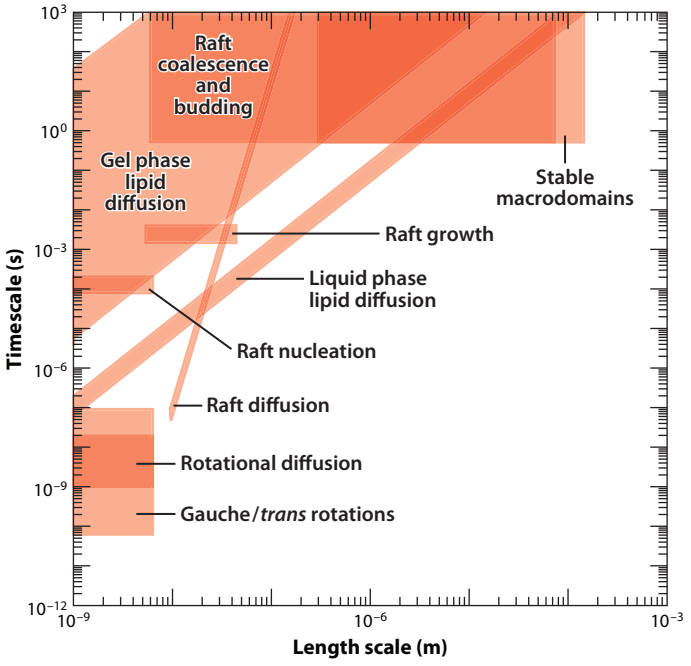

Figure 1

Predicted and observed phenomena in lipid layers span broad length and timescales.

The S_0 phase is typically composed of lipids with saturated hydrocarbon chains in the all-*trans* conformation that can pack in an ordered, compact array. This order is disrupted above T_m by gauche rotations about C–C bonds that prevent compact and orderly packing of the

Fluorescence correlation spectroscopy (FCS): method for measuring diffusion of fluorescent molecules in systems in equilibrium via fluctuations in the number of the molecules in a small open volume

hydrocarbon chains and so lead to formation of the L_α phase. In the L_o state, hydrocarbon chains of the lipid molecules are in extended conformations but nevertheless experience fast lateral diffusion. T_m increases with the length of the saturated hydrocarbon chains. Unsaturated bonds also distort the hydrocarbon chain packing, forming membranes that are fluid under all accessible conditions. The thermodynamics of these phase transformations have been extensively studied (103), making detailed statistical mechanical models possible (70). Other phases can exist even in single-component systems, e.g., subgel and ripple phases seen at temperatures below that of the gel phase and between the gel and fluid phase, respectively (103). Consistent with this structural interpretation of the melting transformation, measurements by fluorescence correlation spectroscopy (FCS) have shown that diffusion is relatively slow in S_0 and rapid in L_α (50).

Bilayers of mixed lipid composition can display phase separation. For example, a binary system containing a high- (h) and a low-melting (l) component can transform from a homogeneous mixture above the T_m of h to a two-phase mixture of solid component h coexisting with liquid component l at temperatures between the T_ms of h and l and then finally to a mixture of two solid components below the T_m of component l (106). Coexistence of liquid phases can be described similarly. Phase diagrams for these and other bilayer membrane systems have been usefully explained and illustrated, although uncertainties arise from experimental inability to detect very small domains (106). Ternary systems of higher- and lower-melting lipids and cholesterol also yield complex and interesting phase behavior (25, 106).

Studying phase transitions and separations yields information about interactions among lipid molecules that are responsible for such important biophysical membrane properties as fluidity/diffusion and mechanical moduli. Membrane phase behavior has been implicated in biological functions such as membrane permeability and ion channel function (8). The strongest biological motivation for studying membrane lipid phase behavior, however, is to better model membrane rafts.

Membrane Rafts

Although the character and even the existence of membrane rafts have been disputed for many years, there is now a consensus on the properties of these specialized membrane nanodomains and their importance for various biological functions (78, 80). Experimental evidence has led to a consensus definition (78, p. 1597): "Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions." Rafts are thought to be in the L_o phase. The historical development of the raft concept as well as descriptions of methods used to measure their properties have been well described (21, 95).

Raft size estimates vary with the measurement technique (1, 43). Domain sizes in the micrometer range have been obtained from confocal imaging of fluorescence-labeled live cells and from measurements of generalized polarization on cells labeled with the fluorophore laurdan (43). A major problem is that rafts are smaller than the resolution limit of the optical microscope, and the trend, over time, has been toward smaller raft sizes (78).

Single-particle tracking analysis of the GPI-anchored protein, Thy-1, yielded a raft size of ~300 nm (90). Measurements of local motion of a bead confined to a laser trap and bound to a membrane protein led to estimated raft sizes in the range of 26 ± 13 nm (79).

Measurements of depolarization of fluorescence by homo-Förster Resonance Energy Transfer (homo-FRET) have been used to analyze interactions among fluorescent glycosylphosphatidylinositol (GPI)-anchored proteins to determine the sizes of clusters on the surfaces of live cells (81, 89). Complemented by theoretical modeling, results indicate that the proteins exist as monomers (>50%) or ~5 nm

cholesterol-dependent clusters of four or fewer protein molecules.

A hop diffusion model derived from high-speed single-molecule tracking measurements suggests that the elementary raft unit is a mushroom-like complex, 1–2 nm in the interior of the membrane and, due to the larger size of the protein ectodomains, 10–15 nm at the surface (51).

At the smallest extreme is a model in which proteins encased in a cholesterol-phospholipid shell containing 15–30 molecules (2 sphingolipids:1 cholesterol) cluster to form lipid domains (1). One criticism is that the lipid shells are reminiscent of boundary lipids that were shown to have only a fleeting existence (51). This model suggests the following question: Do preexisting lipid nanodomains provide a platform for the accumulation of membrane proteins? Or, conversely, do nanoclusters of membrane proteins trigger lipid nanodomain formation? Adopting the latter viewpoint, a new model of raft formation suggests that small mobile L_o domains spontaneously appear and disappear with short lifetimes (<0.1 ms), rather than supposing stable pre-existing L_o nanodomains (36). During their brief existence, these domains can be stabilized by lipid-anchored or transmembrane proteins, perhaps binding to the interface between the lipid domain and the surrounding lipid, acting as a surfactant to stabilize the domain.

Nanodomains in Lipid Bilayer Membranes as Models for Rafts

Characterizing the stability and dynamic properties of nanodomains is crucial to understanding the role of lipid interactions in the formation and function of membrane rafts. This is difficult in natural biological membranes because of their complexity in composition, structure, and active dynamic functions. It is advantageous to study domain formation in simplified model lipid bilayer membranes whose composition can be varied and that are well suited to biophysical techniques including calorimetry, fluorescence microscopy, magnetic resonance, and diffraction methods.

Central to the role of lipid nanodomains as the structural basis of membrane rafts are the following questions:

- Can nanodomains exist in significant (detectable) quantities either in equilibrium or as metastable entities in lipid bilayers of mixed composition?
- What is the distribution of their sizes?
- What are their lifetimes?
- How do these properties depend on the composition of the bilayer membrane?
- Can we understand the equilibrium and dynamic properties of lipid domains, including nanodomains, in terms of the contributions to their free energy of molecular interactions and continuum mechanical properties?

THEORETICAL PREDICTIONS OF THE EXISTENCE AND PROPERTIES OF NANODOMAINS

We begin with descriptions of basic concepts of line tension and growth kinetics, then proceed to summarize several biophysical approaches that have shed light on nanodomains, with an emphasis on the continuum approach (**Figure 2**). Computational approaches including atomistic and coarse-grained approaches are discussed thereafter. Further advances in the theoretical understanding of lipid-protein interactions will likely require interplay between continuum and atomistic approaches (113). However, we emphasize that the primary challenge is experimental validation: Theory currently reaches far ahead of experiment.

Domain Sizes Depend on Line Tension

At the simplest level, the size of a domain is determined by line tension resulting from a positive-free-energy-per-unit length along the phase boundary. The larger this free energy per molecule in the boundary relative to that in the

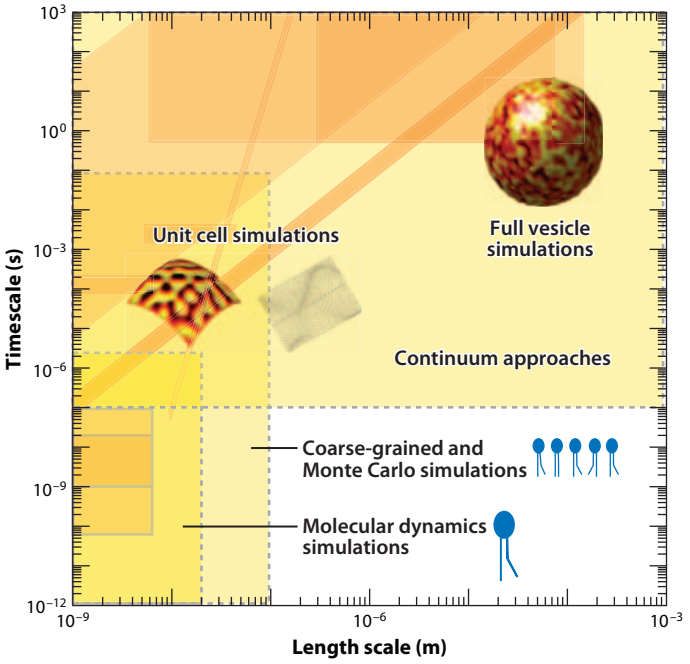

Figure 2

Analytical and simulation approaches span the range of lipid behavior, but simulating over the entire range remains a challenge.

bulk phase, the greater the tendency to minimize the boundary free energy. Hence, if the line tension is large, a system with a few large domains will have a lower free energy (be more stable) than one with many small domains.

Experimental evidence, however, indicates that the factors governing domain size in cell membranes are more complex. Measurements of time-resolved FRET in liposomes composed of a ternary lipid system with higher- and lower-melting lipids and cholesterol indicate that the domain sizes vary with position in a phase coexistence region (18). Domains were smallest near the boundary with the homogeneous L_α phase and largest near the boundary with the L_o phase. A simple model of line tension dictating domain size cannot predict this result unless the line tension depends on the relative amounts of the higher- and lower-melting lipid molecules in the coexistence region.

Clearly, understanding both the origin of line tension and its dependence on membrane composition is essential. The magnitude of the line tension depends on the mismatch between the thickness of the nanodomain and the surround phase (52). Domains in the S_0 and L_o phases are typically composed of longer lipid molecules than the surrounding L_α medium. Indeed, atomic force microscopy (AFM) studies show that rafts are thicker than the surrounding membrane (53, 111). If this increased thickness were uniform throughout the raft, extending to its edge, lipid molecules at the raft boundary would protrude into the water generating a large positive free energy contribution. A more realistic line tension estimate supposes that lipid molecules deform near the domain boundary to minimize elastic and hydrophobic energy, implying that mechanical properties such as spontaneous curvature influence line tension (52). Line tension predicted in this way agrees approximately with estimates from measurements of curvature-dependent domain shapes and budding (5). A study that determined line tension from the power spectra of the fluctuations of domain radii concluded that both line tension and local composition fluctuations varied with the approach of the system to a critical point, as expected for a two-dimensional Ising model (40). Hybrid lipids with one hydrocarbon chain preferring L_o and the other, L_α, could also play a role (10). Although much has been learned, further experimentation is required.

Kinetics of Domain Growth

Under some conditions, lipid nanodomains might be stable at equilibrium or have long but finite lifetimes. Under others, nanodomains might have only a fleeting existence, emerging and disappearing as fluctuations about equilibrium or as intermediates on the pathway to macroscopic phase separation. We review here the kinetic pathway from tiny lipid clusters (nanodomains) to macroscopic domains.

Frolov et al. describe several stages on this pathway following a change of conditions, e.g., temperature, that stabilizes formation of a new phase within an initially homogeneous phase (30). In this initial nonequilibrium state, uniformly distributed lipid molecules are

supersaturated for the formation of the new phase, i.e., some lipid molecules must convert to the new phase to restore equilibrium. The independent growth stage involves nucleation of molecular clusters by fluctuation within the homogeneous medium. Clusters exceeding a critical size continue to enlarge by absorbing surrounding lipids, eventually eliminating the supersaturation condition. Thereafter, two redistribution processes work in parallel: (*a*) merger of mobile domains (dominant process, relatively faster), and (*b*) diffusion of individual lipid molecules from smaller to larger domains, leading eventually to disappearance of the former (Ostwald ripening). At equilibrium, budding-off of nanodomains from larger domains balances growth by merger. When the line tension is low, entropy and boundary energy compete to trap nanodomains in long-lived states. Entropy favors large numbers of small nanodomains; in contrast, boundary energy favors a single large domain. As line tension (boundary energy) increases, nanodomains are eliminated by merger into larger (micrometer-sized) domains. Merger rates could be influenced by repulsive interactions among domains (52). Depending on the size of the nanodomains that arise during the independent growth stage and the magnitude of the line tension, the nanodomains can be relatively stable and might be detected by a suitable physical method.

The Main Lipid Bilayer Phase Transition (MLBPT)

Because the essence of phase separation is the segregation of lipid molecules of different types or conformations into different phases, its explanation must center on differences in interaction energies among these molecules. It is worthwhile to consider a relatively simple problem: the melting phase change that occurs at T_m in bilayers containing only a single type of lipid. The same types of molecular interactions are responsible both for order-disorder phase transformations and for phase separation.

Theoretical work devoted to the MLBPT during the 1970s began with identifying interactions and conformational changes of the lipid hydrocarbon chains—particularly rotations about single C–C bonds—as the main energetic contributions that control the phase transition, and then quantifying this through calorimetry and X-ray diffraction (70). Each C–C bond was taken to have three states: *trans*, gauche$^+$, and gauche$^-$ (29). Taking the all-*trans* conformation of a saturated hydrocarbon chain as the zero energy state, gauche rotations, occurring more frequently with increasing temperature, each add $\varepsilon \sim 0.5$ kcal mol^{-1} to the conformational energy E_{rot} of the hydrocarbon chain (70). Two other intermolecular interactions contribute: van der Waals and excluded volume interactions. Excluded volume interactions are responsible for the substantial cooperativity of the phase transition ensuring that gauche rotations occur in one chain only if gauche rotations in neighboring chains provide space. Although challenging to model, one approach represented the hydrocarbon chain on a two-dimensional lattice with steric exclusion at the lattice sites (69). van der Waals interactions help to hold the bilayer together, balancing centrifugal effects of the excluded volume interactions. Experimental estimates (heat of sublimation of 2 bulk polymethylene chains) predict an enthalpy $E_{att} = \sim 5.5$ kcal mol^{-1} (70). Summing E_{att} and E_{rot} with E_{steric}, the energy of the excluded volume interactions, yields an energy function that, if evaluated for all possible configurations, defines a set of Boltzmann factors, summing to the partition function; from this, one can derive the thermodynamic properties of the system and the probabilities of the different configurations as a function of external conditions. Although the complexity of the model, especially the excluded volume interactions, prevent a general solution, simplified models have been developed that agree reasonably with experimental measurements (69, 70).

The MLBPT can occur in a system with a single lipid component and so requires taking into account interactions experienced by only one kind of molecule. Phase separation

necessarily involves at least two components, and so the range of interactions is more complex. This kind of problem requires more versatile approaches.

The Continuum Theory of Biological Membranes

The first significant contributions to the continuum theory of biological membranes were made independently by Canham (15) and Helfrich (37). These researchers focused on purely mechanical processes to explain the effect of osmotic conditions on membrane conformation in equilibrium, and were evidently influenced by the ideas that guided Singer & Nicolson (96) toward the fluid mosaic model, published roughly concurrently (21).

Consistent with the observation that biological membranes are only a few nanometers thick but have lateral dimensions up to hundreds of microns, the Canham-Helfrich theory neglects the thickness of the bilayer, treating biological membranes as surfaces. Consistent with measurements indicating that the areal stretching modulus is large compared to all other mechanical moduli, the surfaces of the Canham-Helfrich theory are constrained to preserve area. Steigmann et al. (98) discuss the distinction between global and local area preservation in this context. Additionally, because there was then no experimental evidence for heterogeneous distributions of lipids and proteins, the Canham-Helfrich theory is restricted to uniform lateral distributions of lipids and proteins. In combination, these restrictions lead to a special case of a general theory for homogenous, incompressible, fluid surfaces developed by Jenkins (45) [see also Steigmann (97)].

The Canham-Helfrich theory accounts for interactions between neighboring lipid molecules via flexural elasticity; energetic costs due to molecular misalignment are incorporated via two terms. The first has a density proportional to the splay modulus and the square of the difference between the mean curvature and a spontaneous (mean) curvature. No consensus exists regarding the origin of spontaneous curvature: Petrov & Derzhanski (76) attribute it to asymmetry of the membrane molecules, and Beck (6) to packing constraints in the two monolayers [see Safran et al. (85)]. Seifert (88) notes that it might result from differences between the chemical compositions of the constituent monolayers forming a bilayer or to the presence of different chemical environments on the two sides of the membrane. The remaining energy term has a density proportional to the saddle-splay modulus and the Gaussian curvature. Whereas the splay modulus measures energetic costs associated with membrane conformations in which mean curvature deviates from spontaneous curvature, the saddle-splay modulus measures energetic gains or losses associated with conformations in which the principal curvatures may be of opposite sign. Importantly, by the Gauss-Bonnett theorem, the integral over any closed surface of the Gaussian curvature of that surface equals $4\pi(1-g)$, where g is the genus (i.e., the number of holes in the surface). If the saddle-splay modulus is constant, the saddle-splay contribution to the free energy of a membrane of fixed genus is also constant. Thus, for membranes of fixed genus, the only parameters required by the Canham-Helfrich theory are the splay modulus and the spontaneous curvature.

The Canham-Helfrich theory focuses on the midsurface of a bilayer and, thereby, neglects the detailed features of its constituent monolayers. Evans (24) and Helfrich (38) developed extensions of the Canham-Helfrich theory to account for coupling (interdigitation) between monolayers through the addition of an energetic cost proportional to the square of the area difference between the monolayers. Helfrich (38) shows that the additional term leads to the spontaneous curvature of the bilayer becoming nonlocal. Seifert (88) provides an encyclopedic account of results obtained using the Canham-Helfrich theory and its generalizations accounting for interdigitation, including the work of Svetina & Žekš (100), Bozic et al. (9), Wiese et al. (110), Miao et al. (65), and Fischer (28).

Markin (61) extended the Canham-Helfrich theory to account for lateral compositional heterogeneity in a two-component membrane and applied the resulting theory to predict equilibrium shapes and distributions of constituents. This extension amounts to including a bistable concentration dependent contribution to the free-energy density and allowing the splay modulus and/or spontaneous curvature to depend on the concentration. This approach neglects the important effect of line tension on the interfaces separating the two constituents. To account for line tension, one might follow the approach of Cahn & Hilliard (11) and introduce a contribution to the free-energy density proportional to the square of the magnitude of the concentration gradient. Somewhat more recently, Reigada et al. (82, 83), Wallace et al. (108), Campelo & Hernández-Machado (12–14), Funkhouser et al. (31), and Lowengrub et al. (57) have developed and applied dynamical Canham-Helfrich-/Cahn-Hilliard-type theory to study a rich variety of problems related to phase separation. The theory used by these investigators involves a multitude of timescales resulting from the coupling between the mechanical and chemical degrees of freedom in the free-energy density. This coupling generally gives rise to a chemical potential involving several terms. One of these, present in all Cahn-Hilliard type theories, arises from the derivative of the bistable chemical contribution to the free-energy density with respect to the concentration. The other terms arise from dependencies of the mechanical moduli and spontaneous curvature on the concentration. Because the species flux is proportional to the gradient of the chemical potential, these additional terms generate additional effective diffusivities, and, thus, timescales, associated with inhomogeneities in the mechanical properties. One interesting topic that remains to be addressed concerns which of the various timescales govern the earliest stages of phase separation. This topic should be related to unresolved questions concerned with the minimum size of rafts and provide an important linkage to experiment.

Mean Field Approaches

Interactions among molecules are difficult to evaluate. A common simplification replaces a multimolecular system with a single molecule and an external potential that approximates on average the single molecule's intermolecular interactions. This mean field approach is unsuitable for investigating nanodomain size distributions because these depend essentially on differences in the energies of molecular clusters of different sizes and compositions. Nevertheless, a recent mean field examination is instructive (56). This study examines how liquid-liquid phase separations depend on the saturated hydrocarbon chain length of the lipid (54, 84) by comparing the behavior of two-component planar lipid bilayer systems containing a phosphatidylcholine (PC) with two 18-unit long hydrocarbon chains and a PC with two short, $n = 8$–17 unit, chains. The stability of the system was determined by calculating the free energy for a range of mole fraction (x_l) of the long lipid. For $n \leq 12$, plots of free energy versus x_l showed regions of positive curvature, indicating instability and phase separation. Results showed that the greater the length difference between the lipid chains, the greater the range of compositions, x_l, showing phase separation (16). For $n > 12$, the homogeneous lipid mixture was stable overall, but large composition fluctuations existed near values of x_l corresponding to quasicritical behavior. Although Longo et al. argue that nonmacroscopic molecular clusters should have radius <6 nm, the mean field approach cannot directly determine cluster sizes.

PC: phosphatidylcholine (lecithin)

Computational Approaches

A wide variety of numerical methods have been applied to study domain formation and stability in biological and synthetic membranes. At the atomistic level, these include Molecular Dynamics (MD) and Monte Carlo (MC) methods. Coarse-grained simulations model collective membrane phenomena beyond the reach of purely atomistic methods by selectively eliminating details. Studies based

Fluorescence photobleaching recovery (FPR) or fluorescence recovery after photobleaching (FRAP): method for measuring diffusion by observing the recovery of fluorescence in a small area or volume after a photobleaching pulse

on purely continuum-level descriptions treat the bilayer as a thin sheet and consider various free-energy contributions such as membrane bending, membrane tension, and phase boundaries (104). Here, we summarize results and challenges that have emerged from studies applying each of these approaches.

Molecular-level approaches. MD and MC methods have been applied to a wide variety of lipid bilayer properties (35, 99, 112). The challenge in studying raft formation is computational cost: Lipids and cholesterol diffusion in bilayers is too slow to yield significant lateral movement over timescales attainable in state-of-the-art atomistic MD simulations. For example, relatively slow nanodomains of a gel phase in a fluid surround phase could influence the diffusion of lipid molecules both by recruitment of lipids and by forming diffusion barriers in the surround phase, but accommodating these length and timescales is expensive. Strengths of MC simulations are that they can sacrifice detailed description of faster processes to cover broader timescales (cf. coarse-grained approaches, below), and that they can yield verifiable predictions and be calibrated experimentally through measurements of fluorescent lipid probe diffusion, e.g., by fluorescence photobleaching recovery (FPR/FRAP) or FCS.

We focus on a MC study of effects of phase separation and nanodomain formation on the diffusion of bilayer lipid molecules (35). The timescale is set by configurational changes allowable in a single time-step. For diffusion over a molecular dimension, this is calibrated to experimental measurements in an appropriate membrane model; details of faster processes are lost. In a binary system having two components that can each exist in gel or fluid conformational states, distinguished by internal energy and entropy differences from the distribution of *trans* and gauche rotations, three timescales exist: characteristic times for conformational fluctuations of a lipid chain, for diffusion within a gel domain, and for diffusion in a fluid domain (35).

The MC simulation involves lipid molecules disposed on a triangular lattice with the covalently linked hydrocarbon chains of a single lipid molecule occupying adjacent sites. Differences of interaction energies between lipid types and conformations determine phase behavior. Free energy for a specific configuration involves conformation-dependent energies of individual molecules and interaction energies of adjacent molecules, determined from calorimetry of single-component membranes and from phase diagrams of two-component systems. Prospective MC steps involve random configuration changes (molecular conformational state change or diffusion involving lattice position exchanges with neighboring lipid molecules); changes are accepted if the associated Gibbs free-energy change δG satisfies $\exp[-\delta G/k_B T] \leq R$, where R is a random number between 0 and 1. R is reduced to account for lower mobility in gel domains or coexisting gel and fluid domains [Sugar (99) achieved this using free volume theory]. After many steps, systems initially in nonequilibrium, e.g., all-*trans* states, approach equilibrium distributions of molecular states and positions. Further cycles provide molecular distributions that reveal spontaneous fluctuations and lipid domain formation.

Simulated FCS measurements predicted fluorescence intensity fluctuations from simulated fluorophores diffusing randomly through a stationary Gaussian excitation intensity profile (35); the associated fluorescence fluctuation autocorrelation function yields the diffusion coefficient (23). In pure fluid and gel phases, both experimental and simulated FCS autocorrelation curves were consistent with simple diffusion. In systems with coexisting gel and fluid phases, however, a simple superposition of the diffusion in the pure phase domains could not account for the correlation curves (35), perhaps owing to changes of state of the lipid molecules during their traversal of the intensity profile. However, the data are insufficient to draw definite conclusions.

At temperatures slightly lower and higher than those at which macroscopic phase

separation occurred, small fluid domains were observed in the gel phase and small gel domains in the fluid matrix, respectively (35). Hence, nanodomains may most readily be seen in the wings of the phase separation transition, where they might exist either stably due to a balance of line tension and electrostatic dipolar repulsion (49), or transiently as intermediates along the path to macroscopic phase separation. Nanodomain lifetimes, which could in principle be measured by FCS using fluorophores exhibiting enhanced fluorescence intensity in nanodomains compared to the surround phase, provide another potential tie between simulation and measurement.

Coarse-grained simulations. The MC/MD diffusion problem has motivated the development of coarse-grained, multiscale, and continuum-based methods for studying domain formation in lipid bilayers over much greater temporal and spatial scales. Coarse-grained simulations have been applied to study the kinetics of phase transition (62) and phase separation (91) in multicomponent membranes. Techniques for coarsening MD models include coupling to continuum representations of the membrane, and the elastic modulus is obtained via nonequilibrium MD or dissipative particle dynamics (2, 3) (for detailed reviews of other methods see References 67 and 74). Importantly, recent coarse-grained simulations by Eric Jakobsson's group have been used to predict phase diagrams and specific heats for DPPC/cholesterol mixtures in bilayers that are consistent with the experimental observations (72, 73). Rather than relying on pseudoatomistic methods, their approach is based on equilibrium statistical models developed by Marcelja (60) and the time-dependent Ginzburg-Landau equation for dynamics. The extension of the approach to ternary systems is described by Pandit & Scott (74). Validating dynamic coarse-grained predictions is challenging because molecular-level details relating to fluorescence fluctuations can be masked.

Continuum-level simulations. Continuum-mechanical models largely build on the Canham-Helfrich-Evans theory for homogeneous biomembranes. The classical equilibrium equations (46) are fourth-order nonlinear partial differential equations (PDEs) that are nontrivial to solve. Recent numerical approaches have employed finite elements with subdivision surfaces (27) and phase-field regularizations that represent the membrane as a diffuse interface (20).

Although most continuum-based studies have focused on experimentally verifiable equilibrium shapes of homogeneous bilayers, recent efforts have considered multicomponent vesicles (58) and composition dynamics (57, 109). These models yield rich, experimentally verifiable, multicomponent vesicle configurations (109) and qualitative insight into phase separation dynamics. Studies of configurations far from equilibrium conclude that phase separation via spinodal decomposition and coarsening is closely coupled to vesicle shape (57). At the level of nanodomains, continuum simulations can yield spatial distributions of lipid species that may be verifiable by FCS.

DPPC: dipalmatoyl phosphatidylcholine, or dipolmatoyl PC

EXPERIMENTAL DETECTION AND CHARACTERIZATION OF LIPID NANODOMAINS

Understanding the physics of nanodomains requires determining how their sizes and lifetimes vary with membrane composition and curvature as well as external conditions such as temperature. Challenges include the optical resolution limit and the stochastic nature of even simplified systems such as giant unilamellar vesicles (GUVs) (59, 86). The field has relied on indirect methods including diffusion measurements and FRET, but high-resolution imaging methods that could resolve rafts more directly, such as near-field scanning optical microscopy (NSOM), super-resolution fluorescence microscopy, and AFM, offer potential, as do methods based on fluorescence fluctuations. Although nanodomains can be detected and even visualized in lipid membranes,

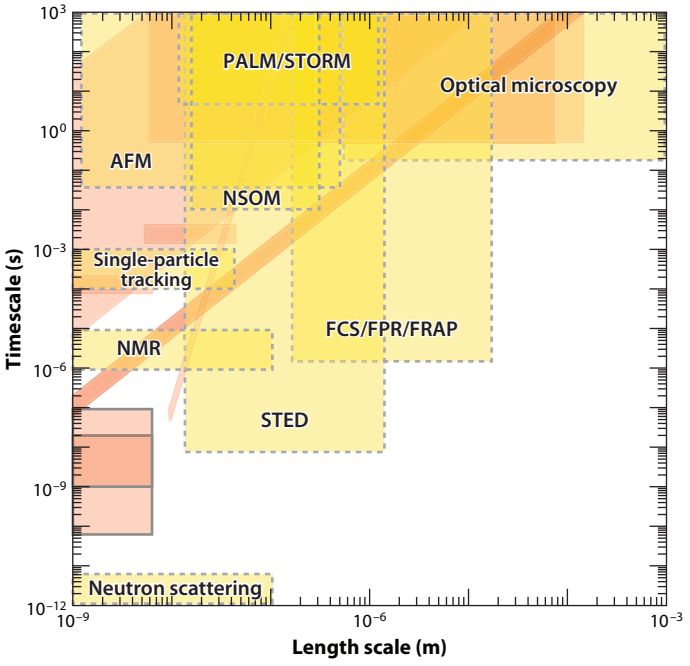

Figure 3

Experimental techniques cover most of the range of lipid behavior but still do not adequately determine nanodomain sizes and lifetimes. Abbreviations: AFM, atomic force microscopy; FCS, fluorescence correlation spectroscopy; FPR, fluorescence photobleaching recovery; FRAP, fluorescence recovery after photobleaching; NMR, nuclear magnetic spectroscopy; NSOM, near-field scanning optical microscopy; PALM, photoactivated localization microscopy; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy.

no existing methods are entirely suitable for determining the distribution of nanodomain sizes and their lifetimes (**Figure 3**).

FRET

FRET has provided important information about the clustering of GPI-anchored membrane proteins (89) and of lipids (26). The former study used homo-FRET, energy transfer between identical molecules that depolarizes fluorescence without changing fluorescence intensity. FRET occurs only if the donor and acceptor molecules are within the Förster radius, R_0, typically ~10 nm. From their measured average density, the observed extent of homo-FRET of fluorescent GPI-anchored proteins was possible only if they were clustered (89). Anisotropy decay rate measurements indicated that the fluorescent proteins were in very close proximity. Combining photobleaching to change the density of fluorescent proteins with mathematical modeling demonstrated that the protein clusters were smaller than R_0. This powerful approach might be applicable also to study the organization of lipids in nanodomains. Fluorescent lipids would have to be present at relatively low concentration in the membrane and therefore would have to have a strong tendency to partition into the nanodomain from the surround phase.

Other applications of FRET have detected nanodomains too small to be resolved by light microscopy. In one, a pair of donor lipids—one incorporating selectively into disordered and the other into ordered domains—are in the presence of an acceptor that selectively partitions into disordered domains (93). In a homogeneous system the quenching of both donors is comparable, but in bilayers with separated ordered and disordered phases, the donor in the disordered phase is more efficiently quenched than the one in the ordered phase. Another approach uses a pair of donor and acceptor lipid probes that partition into different membrane phases; phase separation therefore separates the lipid probes and reduces FRET efficiency (26).

Other fluorescence methods including anisotropy and lifetime measurements have been used to develop phase diagrams for ternary systems (17, 19). The fluorescence of the lipid probe laurdan (6-dodecanoyl-2-dimethylaminenaphthalene)—sensitive to water exposure and thus to the membrane phase state—has been used to detect phase coexistence in GUVs (4).

Mobility Measurements

Mobility has been used to probe for nanodomains (35) and to study rafts in cell

membranes (47). FPR/FRAP in the plasma membranes of several cell types shows that domain connectivity affects diffusion of fluorescent proteins (63). At 25°C proteins commonly associated with rafts diffused rapidly and with complete fluorescence recovery in the apical membranes of epithelial cells. Other membrane proteins not typically associated with rafts diffused 3–4 times more slowly with only partial recovery, indicating that the raft proteins but not the nonraft proteins could percolate throughout the membrane in a continuous raft-like phase. In a related study, electron spin resonance (ESR) measurements on spin labeled lipids in live cells suggest that the L_o phase is a major topologically continuous domain component of the membrane (101).

Single-Molecule Tracking

A single-molecule method for measuring the viscous drag on raft and nonraft proteins in cell membranes involves a microbead attached to a membrane protein that is confined to a small area via laser trapping (79). Bead motion was tracked with subnanometer and microsecond accuracy. Confinement allows the motion of the attached membrane protein to be sampled over a very small spatial range evading obstruction by membrane barriers. Hence, measured diffusion rates of transmembrane and GPI-linked membrane proteins depend on membrane lipid viscosity. However, for membrane proteins embedded in rafts/nanodomains, diffusion rates are independent of membrane anchor type and are less than for nonraft proteins. Raft sizes ($r = 26 \pm 13$ nm) were estimated using the Saffman-Delbrück scaling [diffusion coefficient $D \sim \log(r)$]. However, recent studies suggest stronger scaling: $D \sim r^{-1}$ (32, 71) or above $r \sim 7$ nm for a typical membrane, $D \sim r^{-2}$ (33).

Nonoptical Methods

NMR was used to detect phase heterogeneity in membranes. Domain sizes on multilamellar vesicles obtained by NMR (~80 nm) were smaller than those observed by fluorescence on GUVs (>1 μm) (107). The diameter of the domains was approximately determined from the diffusion coefficient of the lipid molecules and their lifetime in the domains.

Suboptical Resolution Methods

AFM. Imaging methods that evade the optical diffraction limit may provide the needed information about the sizes and lifetimes of nanodomains. AFM is well suited to characterize sizes of rafts in cells and nanodomains in bilayer model membranes (7, 68, 111). A comparative study of nanodomains visualized using AFM and predicted using FRET in a ternary system is instructive. Regions of the cholesterol/dilauroyl PC (DLPC)/DPPC phase diagram where FRET donor and acceptor fluorophores partition into different phases, but where no phase separation is visible by fluorescence microscopy, have been explained through nanodomain phases too small to be resolved by optical microscopy (26). AFM measurements on the same ternary system in the form of supported lipid bilayers (102) show domains enriched in DLPC and DPPC, as distinguished by their thicknesses (4.33 and 5.51 nm, respectively) in the region of the phase diagram where no macroscopic domains could be seen optically. However, AFM is too slow to observe domain dynamics.

NSOM. NSOM provides images with resolution below the conventional optical limit using an optical probe that emits an evanescent wave to excite fluorescence from a very small region of the sample. Scanning across the sample yields intensities, each from a pixel that is small compared to the wavelength of the exciting light, from which a high-resolution image is reconstructed. Images of fluorescence-labeled plasma membranes of fixed fibroblasts, dried or in buffer, reveal fluorescent lipid probe clustering suggestive of lipid domains, but membrane protein clusters were not correlated with

the fluorescent lipid patches (41). Despite the promise of this approach, no systematic applications have yet studied lipid domain organization in cells or model membranes.

Super-resolution. Several techniques exist for super-resolution optical imaging (34). Stimulated emission depletion (STED) microscopy employs a light pulse to deplete the excited states and thereby suppress fluorophore emissions in a ring around the focal point of a microscope, reducing the excited region to below the resolution limit. Scanning provides super-resolution images. Photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) reconstruct super-resolution images from precise localizations of individual fluorophores; single fluorophores can be located to nanometer resolution if enough fluorescent photons can be collected from excited fluorophores spaced beyond the resolution limit. These methods have been extensively employed to study membrane protein organization but have been little used to study membrane lipid organization. One study of nanoscale membrane lipid dynamics applied STED and FCS to measure lipid diffusion in nanoscale areas of a membrane (22): In contrast to phosphoethanolamine (PE), which diffused normally, sphingomyelin (SM) displayed both slow and fast diffusion. Slow diffusion was attributed to transient trapping of SM molecules by membrane structures. Depletion of cholesterol from the membrane eliminated the slow diffusion mode. These results are consistent with the transient association of SM but not PE with cholesterol-containing raft-like structures.

FCS. Fluctuation spectroscopy based on FCS and later extensions could provide powerful tools to determine nanodomain size and lifetime distributions. These methods determine the brightness (emitted photons per particle per second) of fluorescent molecules or aggregates. An important advantage is that in the absence of intermolecular quenching or enhancement, brightness varies linearly with aggregate (nanodomain) size, a stronger dependence than provided by measurements of diffusion. Fluorescence fluctuation amplitudes interpreted through the photon count histogram provide the basis for measuring numbers of nanodomains and their brightnesses (48, 66). This requires the number of fluorophores binding to a nanodomain to increase with its size. Measurements of nanodomain lifetime require that lipid probe fluorescence differs depending on whether it resides in a nanodomain or in the surrounding phase. These methods have yet to prove their effectiveness in studies of lipid nanodomains.

PROSPECTS FOR THE FUTURE STUDY OF NANODOMAINS IN MODEL BILAYER MEMBRANES AND THEIR RELATIONSHIP TO MEMBRANE RAFTS

To test whether lipid nanodomains play a driving role in the formation of membrane rafts, it is essential to determine nanodomain sizes and lifetimes in simple model membranes of varying lipid and protein compositions. Can nanodomains of appropriate sizes be stable for time intervals long enough to provide platforms for binding specific membrane proteins? If not, then rafts would have to arise from a concerted interaction of proteins and lipids (36).

Theory predicts the characteristics of nanodomains in simple model membranes as well as their dependence on membrane mechanical properties such as curvature, but experimental methods still do not adequately determine nanodomain sizes and lifetimes. Although AFM is the imaging approach perhaps best developed for this purpose, it may not be able to capture the possibly rapid kinetics of nanodomain appearance and disappearance. New super-resolution and fluorescence fluctuation methods hold great promise but have yet to demonstrate their abilities to characterize fluctuating nanodomains.

SUMMARY POINTS

1. Although membrane rafts likely contribute to many physiological and pathophysiological cell functions, the physical and chemical factors that determine the sizes and lifetimes of these important structures are not yet well understood.
2. Key open questions involve membrane raft stability and its dependence on membrane composition. Measurements of the sizes and lifetimes of lipid nanodomains in model bilayer membranes can provide important information about these characteristics.
3. Nanodomains might exist only as transient structures on the pathway to the formation of macroscopic phase domains or they might exist stably in equilibrium or metastably with long lifetimes. Both theory and experimental measurements are needed to explore these possibilities.
4. A wide variety of theoretical approaches are required to study nanodomain properties, including continuum-level methods based on Canham-Helfrich-Evans theory and molecular level simulations using Monte Carlo and mean field approaches.
5. Theory has defined conditions under which nanodomains in lipid bilayer membranes might exist stably in equilibrium or as transient clusters on the pathway to macroscopic domains.
6. Experimental measurements of the size distribution of nanodomains and their lifetimes lag behind these theoretical predictions.
7. Methods including AFM (atomic force microscopy), NSOM (near-field scanning optical microscopy), and super-resolution methods (STED, PALM, and STORM) as well as fluorescence fluctuation methods based on FCS have the potential to provide information about both equilibrium and dynamic properties of nanodomains.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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89. Uses homo-FRET to demonstrate that selected cell surface proteins are clustered into cholesterol-dependent raft-like structures.

102. Provides a detailed phase diagram for a common ternary system that includes regions in which nanodomains are thought to be stable.

106. Reviews phase behavior in GUVs with mixed lipid composition.

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