Lipid Rafts: Feeling is Believing
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In the late 1990s, accumulated evidence led to the proposal that biological membranes are composed of microdomains of different lipids, which form functional “rafts.” Recent work using atomic force microscopy has given us new insights into the factors influencing the formation and behavior of these physiological microenvironments.

In the late 1990s, evidence that had accumulated since the 1970s led to the proposal that biological membranes are composed of microdomains of different lipids, which form functional “rafts” (17). The physiological relevance of this concept was initially somewhat controversial but is now much less so. The treatment of membranes with nonionic detergents leads to the production of detergent-resistant membrane fragments that are enriched in cholesterol and sphingolipids, such as sphingomyelin. These are believed to represent the biochemical equivalents of rafts. Furthermore, specific proteins are confined to sphingolipid membranes, leading to the proposal that rafts might be involved in cellular processes such as cellular signaling and protein trafficking (18), and in recent years it has become evident that atomic force microscopy (AFM) provides opportunities to examine closely the structure of cells, their membranes, and associated proteins. Most recently, AFM has proved an admirable technique to examine both the lipid raft microdomains and the proteins that inhabit them.

The atomic force microscope is a scanning probe microscope. It produces information by drawing a very sharp probe in a raster pattern across a surface and building up an image as a result of the vertical deflections of the probe each time it encounters some obstacle as it sweeps back and forth, effectively “feeling” its way across the surface. This sounds like a rather crude technique, but a combination of materials science, microelectronics, and well-designed software means that AFM can produce images of exquisitely high resolution (<1 nm). In addition, AFM has the capability of operation under fluid, meaning that it is possible, in contrast to electron microscopy, to perform experiments on biological specimens and to produce images under near-physiological conditions. The experimental conditions can be designed to conduct experiments on cultured cells in vivo, or, equally, biological macromolecules can be examined either in their native membranes or reconstituted in an artificial system, and the behavior of these macromolecules can be examined as they interact with other macromolecules. It is also possible to perform manipulations, such as adding ATP to the system, and then observe the effect of these manipulations on the macromolecules under study. For these and other reasons, AFM was adopted by biologists very soon after its development in 1986.

The biological applications have grown since then. This has been concurrent with the refinement of AFM technology, allowing even higher image resolution and the gathering of information about forces of interaction between molecules (20). The early practitioners of biological AFM held out high hopes for the investigation of membrane features on intact, living cells. Indeed, much useful data has been produced from AFM of cells in vivo, including, for example, information clarifying the exocytotic or endocytic process (15) or demonstrating changes in nuclear membrane pore structure in response to ATP (12). However, intact membranes in situ present problems, because they often contain numerous structures, many of which may be indistinguishable by AFM. This being the case, several laboratories began in the mid 1990s to use AFM to study isolated and/or purified membrane proteins. The experiments present their own problems. Many membrane proteins are difficult to express in appropriate quantities, and they often possess hydrophobic domains that normally span the membrane. Therefore, although it is possible to use AFM to look at membrane proteins immobilized on some suitable surface, the fact that the molecules are not in their native environment inevitably gives limited structural information.

Interest has therefore turned recently to looking at membrane proteins in some sort of either natural or artificial lipid environment. One approach is to use systems in which large quantities of particular membrane proteins (mainly bacterial proteins) may be stabilized. Engel, Müller, and co-workers in Basel were early practitioners of this technique, building on earlier experience of X-ray crystallography, and they produced (among other things) innovative AFM data revealing molecular features of bacteriorhodopsin in purple membranes (11). The same group has studied eukaryotic systems, including two-dimensional crystals of human aquaporin-1 (9) and rhodopsin (2). Very recently, they have produced images of single sodium-driven rotors from Ilyobacter tartaricus ATP synthase embedded in a lipid membrane under a physiological solution (10). Time-lapse AFM images showed the movement of single proteins within the membrane, a topic of fundamental importance because there is much interest in the physiological significance of trafficking of proteins within and
Most eukaryotic membrane proteins are, however, not amenable to production in the large quantities required for the production of two-dimensional crystals. Under these circumstances it is still possible to use AFM to reveal structural information, provided that it is possible to isolate sufficient material and, crucially, to ensure the purity of that material. Such an approach was taken by Geisse et al. (3), who studied the 16-kDa protein syncollin, which is associated with the luminal membrane of the zymogen granule and appears to be of significance in exocytosis in the exocrine pancreas. Syncollin has interesting biochemical characteristics; it evidently forms oligomers (which may be dissociated by exposure to reducing agents), and it is soluble at high but not at low pH. The association of syncollin with the granule membrane also requires cholesterol. The authors combined AFM with biochemical techniques and electron microscopy. This latter technique suggested that syncollin exists as doughnut-shaped structures, and the biochemical work revealed that it was likely to exist as homooligomers. Furthermore, addition of syncollin to liposomes appeared to make them permeable to low-molecular-weight fluorescent dyes. The AFM work complements the other data, revealing a structure that is composed of six monomers (Fig. 1). Biochemical experiments indicated that syncollin breaks down into its constituent monomers either at pH 11 or in the presence of the reducing agent dithiothreitol (DTT). By using AFM it is possible to measure the volume of protein particles, and the volume is directly related to the proteins’ molecular weights (16). Geisse et al. (3) prepared samples of syncollin and imaged them by AFM at pH 11 or in the presence of DTT, as well as under control conditions. The structures, as expected, dissociated under the appropriate conditions, consistent with previous evidence that such treatments lead to the dissociation of the syncollin homooligomer into monomers.

The investigation by AFM of purified proteins in their native membranes is valuable, but concurrently with the refinement of techniques of AFM the concept of lipid rafts came to the fore. In mixed lipid bilayers, sphingomyelin/cholesterol-enriched rafts are thought to exist in a liquid-ordered state in which the lipid molecules are tightly packed but still able to diffuse laterally. The formation of these liquid-ordered phase domains is encouraged by the presence of cholesterol, which is surrounded by a liquid-disordered phase (composed principally of phospholipids with unsaturated and, therefore, kinked fatty acyl chains), which allows for a greater degree of lateral diffusion.

Recently there has developed great interest in studying the behavior of lipid rafts in bilayers of defined lipid composition. Such studies have often used fluorescence microscopy to monitor the distribution of fluorescent raft markers (1, 13). The resolution of this technique is such that the domains seen are large (several microns), which calls into question their physiological significance. Recently, however, it has become clear that AFM represents an ideal tool with which to study raft behavior. The kinked structure of the fatty acyl chains of phospholipids results in a shorter molecular length relative to the straight sphingomyelin molecules: a bilayer composed of phosphatidylcholine is 3.5 nm thick, whereas a bilayer composed of sphingomyelin is 4.6 nm thick (19). Hence, proteins should protrude from the nonraft background by ~1 nm, a difference in height that is clearly discernible by AFM. Accordingly, several groups have seized the opportunity to use AFM to examine this potentially important topic. In epithelial cell plasma membranes are polarized into apical and basolateral domains: the apical membrane is rich in sphingolipids, and the basolateral membrane is rich in the unsaturated glycerolipid phosphatidylcholine. To study the propensity for raft formation in the brush border of renal epithelial cells, Milhiet et al. (7) deposited lipid monolayers on mica, the lipid composition mimicking that of the outer leaflet of the renal brush border (apical) membrane. It was found that the proportion of monolayer accounted for by the sphingomyelin-enriched liquid-ordered phase varied according to the ratio of sphingolipids to glycerolipid comprising the monolayer. Furthermore, when cholesterol was added to the mixtures at varying proportions of the total lipid, striking alterations in the topology of the monolayers were observed. As the proportion of cholesterol increased, the relative areas of raft domains also

**FIGURE 1.** Top: isolated membranes containing syncollin purified from rat zymogen granules. The atomic force microscopy (AFM) image shows bilayers (green/brown) of ~4 nm thickness laid down on a mica substrate (blue). The protein complexes protrude from the bilayer (white). Bottom: lipid-associated syncollin, seen under higher resolution, adopts a doughnut-shaped structure. This high-magnification, 3-dimensional AFM view of a bilayer-associated syncollin molecule illustrates the 6 oligomers making up the structure, surrounding the pore. The image was produced by imaging supported membranes containing syncollin, and the sample was imaged under HEPES-buffered saline. The outer diameter of the oligomer is ~31 nm, the pore diameter ~6 nm. More details are given in Ref. 3.
increased, but after a reaching a crucial level of cholesterol (exceeding 20 mol%), a progressive reduction of the size of raft domains was seen. The fact that the lipid composition of the model renal membrane would appear to be so significant is of potential physiological importance because the function of membrane proteins located in the structurally different apical or basolateral membranes of cells is likely to be influenced by temperature. This may in turn have an effect on, or be affected by, the lipid phase in which the proteins find themselves (5, 7).

The effects of changing concentrations of cholesterol shown in Ref. 7 were reflected in the findings of another group who used AFM to study the effect of cholesterol on monolayers and bilayers composed of sphingomyelin and dioleoylphosphatidylcholine (DOPC) (4). As shown in Ref. 7, higher levels of cholesterol led to suppression of sphingomyelin domains and coalescence of the lipid layer. Note that the raft diameters seen in these AFM studies (on the order of 100 nm) are much smaller than those seen in the fluorescence-based studies and are therefore more likely to reflect the behavior of rafts in biological membranes.

An innovation in Ref. 4 was the production of AFM images in real time as cholesterol was introduced (by addition of water-soluble cholesterol at the beginning of a scan followed by capture of sequential images) or removed from the supported lipid layer (by introducing the cholesterol-sequestering agent methyl-β-cyclodextrin to the system and following the form of the bilayer during the course of a scan). These manipulations have striking effects on the rafts' topology (Fig. 2).

Addition of water-soluble cholesterol to a bilayer initially containing 10 mol% cholesterol resulted in an increased size of the sphingomyelin-rich domains with an eventual formation of a single raft-like lipid phase. In contrast, depletion of cholesterol led to a disappearance of discrete raft domains. This is intriguing, since sphingomyelin-rich domains were evident in sphingomyelin/DOPC bilayers before addition of cholesterol, so why should they not exist once the bilayers have been depleted of cholesterol? It is possible that the removal of cholesterol from a bilayer leaves “holes” in the bilayer, which creates a drop in lateral pressure, leading to dissolution of the lipid domains (4). Alternatively, cholesterol removal might cause the sphingomyelin-enriched domains to dissolve into the surrounding fluid phase, and subsequent formation of gel-phase domains by sphingomyelin may be sufficiently slow as to be undetectable over the time course of the experiments.

What could be the physiological relevance of the preferential location of certain proteins in rafts? Simons and Toomre (18) suggest that localization and protection of cell signaling processes may be a significant reason. Rafts may effectively act as platforms for individual receptors. If receptor activation takes place in a lipid raft, the signaling complex is protected from nonraft enzymes such as membrane phosphatases that might otherwise affect the signaling process. Furthermore, raft binding recruits proteins to a new microenvironment, where phosphorylation may be modified by local kinases and phosphatases, resulting in downstream signaling (18). In addition to a potential role in cell signaling, protein localization to rafts also appears to be involved in intracellular trafficking. Proteins that show affinity for a sphingolipid environment include glycosylphosphatidylinositol (GPI)-anchored proteins, acylated proteins, and palmitoylated transmembrane proteins (18), probably because they possess saturated acyl chain membrane anchors that associate preferentially with the liquid-ordered domains. As would be expected from the polarized localization of sphingolipids in the plasma membrane of epithelial cells (see above), it has been shown that GPI-anchored proteins are delivered specifically to the apical sur-

![FIGURE 2. Top: effect of cholesterol addition on lipid rafts in supported lipids bilayers imaged under HEPES-buffered saline. A: sphingomyelin/dioleoylphosphatidylcholine (DOPC; 1:1 mol/mol) bilayer containing cholesterol (~10 mol%). Water-soluble cholesterol (50 μg/ml) was added, and the system was allowed to equilibrate briefly before images were captured sequentially (B–F). All images are 4.5 × 4.5 μm; scale bar is 1 μm. The directions of the horizontal streaks on the images represent physical disturbances that took place as cholesterol was added. Bottom: effect of cholesterol depletion from a cholesterol-saturated supported bilayer. A: supported lipid bilayer followed treatment with water-soluble cholesterol. Methyl-β-cyclodextrin (MCD; 20 mM) was injected while scanning (scanning progressed from bottom to top); the disturbance as the addition takes place is evident at the bottom of the scan. Initially, a high-cholesterol bilayer was evident, exhibiting no lateral heterogeneity. After ~2 min, discrete rafts were observed, and then, following further cholesterol extraction, the rafts “melted” into the surrounding fluid bilayer. Images are 3 × 3 μm; scale bar is 500 nm. Figure reproduced from Ref. 4, with permission.](http://physiologyonline.physiology.org/doi/abs/10.1152/physiolrev.00089.2004)
face of these cells (6).

It is clearly important to understand the process by which proteins partition into rafts. To help achieve this goal, AFM has been used to examine the relationship between the GPI-anchored protein alkaline phosphatase and a supported bilayer containing sphingomyelin-enriched domains. When added to a preformed sphingolipid/DOPC bilayer, intestinal alkaline phosphatase showed preferential insertion into the sphingomyelin domains, mainly appearing at the periphery of the raft (8); however, when the bilayer also contained cholesterol, the alkaline phosphatase was seen to be homogeneously distributed in the rafts. Cleavage of the GPI anchor from the alkaline phosphatase, by using phospholipase C, abolished the appearance of alkaline phosphatase molecules in the rafts (8). The homogeneous distribution of alkaline phosphatase in the rafts containing cholesterol was hypothesized as being associated with cholesterol’s property of encouraging the protein to pass into the liquid-ordered phase. In contrast, when liposomes containing placental alkaline phosphatase (PLAP) were used to produce supported bilayers, the PLAP was found to become localized in a homogeneous fashion to the sphingomyelin rafts irrespective of the presence or absence of cholesterol (14). PLAP appeared as protrusions from the membrane (Fig. 3), and its identity was confirmed as a PLAP dimer by measurement of the particles’ dimensions and calculation of molecular volume (16).

The first steps have therefore been made in using AFM to study the interactions of individual proteins with lipid microdomains. More sophisticated experiments, involving more than one protein species, can easily be envisaged. This is all the more enticing because the role of rafts in cell signaling, although now less controversial, remains unclear. Simons and Toomre (18) indicate that receptors involved in the induction and control of signaling could behave in three different, although perhaps complementary, ways in rafts. Those receptors that are associated at steady state with rafts could be activated through ligand binding. Receptors with a low affinity for rafts could, once bound to their ligand, oligomerize, and this could lead to an increased time of residence in rafts. Thirdly, activated receptors could recruit cross-linking proteins that bind to proteins in other rafts, and this could lead to coalescence of raft domains. The occurrence of these clusters of signaling molecules and associated proteins could result in amplification through physical apposition and interaction of signaling molecules. All of these possibilities lend themselves, to some extent, to investigation by AFM, so the prospects for future experiments are exciting.

References


