Bilayer Membrane Interactions with Nanofabricated Scaffolds

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Introduction

Cells and their internal compartments are enveloped by membranes based on lipid bilayers. The bilayer serves as host to hundreds of embedded and associated proteins that impart diverse functions, including selective transport, signal transduction, cellular recognition and locomotion.¹ Most membrane lipids and proteins diffuse freely in the plane of the membrane, described classically as a fluid mosaic.² The membrane is not just a simple permeability barrier, but a highly complex, dynamic and multifunctional structure. The spatial organization of lipids and proteins in biological membranes is regulated not only by specific molecular interactions with neighboring biomolecules, but also by the structural and mechanical properties of the membrane, such as bilayer thickness, intrinsic curvature, elastic moduli, and lipid composition.³ Understanding the lateral structure of membranes and how it relates to function is critical to advancing biological science and technology. It has become increasingly apparent that nanoscale

structure and organization in cell membranes play significant roles in cell signaling and membrane physiology.^{4,5,6,7,8,9}

Separation of lipids into distinct domains of greater order (lipid rafts) and anchoring to the cytoskeleton are two main mechanisms for organizing the membrane in living cells, features which have proven difficult to reproduce in traditional model membrane systems (e.g., unilamellar vesicles, black lipid membranes, and supported bilayers). Certain mixtures of synthetic lipids have resulted in easily observed, stable microscopic domains.¹⁰ Synthetic lipid mixtures that give rise to nanoscopic domains have also been discovered.¹¹ However, these domains are not visible in optical microscopy and are believed to have a fleeting existence, making them difficult to study. The fact that the measured diffusion coefficients of many membrane proteins are far smaller than expected implicates interactions with either the cytoskeleton directly or through intervening proteins.¹² Both lipid rafts and membrane-cytoskeletal interactions involve the formation of nanoscale structures. In many cases, these nanostructures are transient, further frustrating efforts to study them.

Advances in micro/nanofabrication, however, have resulted in the development of increasingly realistic synthetic models of membrane-cytoskeleton interactions, enabling the discovery of design principles governing biological membrane formation and organization, the creation of increasingly complex hybrid micro-nanostructures comprised of both biological and non-biological components, and the emergence of functionality at the nanoscale.¹³

This review will describe how established model bilayer systems, such as liposomes, supported lipid bilayers, and suspended bilayers, can be configured to adopt features reminiscent of real membrane-cytoskeleton interactions. Similarly to a real cytoskeleton, a micro/nano-fabricated scaffold should be able to nucleate domains in the membrane and impart order at

defined locations, while allowing free diffusion in the bulk of the bilayer. A realistic membrane model should thus have well-defined lateral structure and distributed attachment points to an underlying substrate, functionalized as needed to tailor the surface chemistry. Diffusion coefficients in such a model will decrease with increasing observation times, corresponding to free-space diffusion of molecules within domains at early times, followed by obstruction of diffusion by the barriers between domains at later times.¹³

A number of comprehensive reviews have been published on various aspects of lipid bilayers, including their formation mechanisms, characterization techniques, their regulation of membrane proteins, and their integration in biosensors and other devices.^{3,11,13,14,15,16,17,18,19,20,21,22} Owing to space limitations, this review will focus exclusively on micro/nanostructured scaffolds that emulate in some way the connections of a cellular membrane to an underlying “cytoskeleton” (figure 1). These structured scaffolds could be fabricated from solid-state inorganic materials like metals,^{23,24,25} semiconductors²⁶ or oxides,^{27,28} “soft” polymeric biomolecules^{29,30,31} such as PEG, collagen, or actin, or combinations of these. Recent work on micro/nanoporous scaffolds that can also be classified as model systems for understanding membrane-cytoskeleton interactions will be described.

Molecular-based “scaffolds”

Supported lipid bilayer membranes self-assemble on hydrophilic substrates in aqueous solution on a thin (1 to 2 nm thick) lubricating layer of water, which imparts lateral fluidity to the membrane and helps to stabilize the bilayer, but also impedes the incorporation of transmembrane proteins due to steric repulsion with the solid substrate (figure 2).³² Supported bilayers can be modified however to include spacer molecules that can decouple the bilayer from the substrate, such as organosilanes on silica, alkylthiols on gold, tethered lipid monolayers, or

cushioning polymers (PEG-lipids), hydrogels, or polyelectrolyte films.¹⁸ Spacer molecules typically consist of a hydrophobic group that binds to the bilayer, a hydrophilic backbone, and a coupling moiety for tethering the spacer to the solid substrate.

The surface chemistry of molecular scaffolds can be precisely engineered to control the coupling of the bilayer to the surface, the spacer molecular length and shape, and the chemical functionality along the backbone, such as degree of hydrophobicity or hydrophilicity, the inclusion of saturated versus unsaturated bonds, and the presence or absence of bulky side groups.³³ Examples include linkers that can bind to silanol groups on silica to form siloxane linkages,³⁴ the formation of amide bonds at the substrate surface due to reaction of amine-coated glass with carboxylate groups or *N*-hydroxy succinimide (NHS) esters,³⁵ coupling of tethered biotin with streptavidin,³⁶ or spacers with pendant nickel nitrilotriacetic acid (Ni-NTA) groups for bonding molecules with polyhistidine tags.³⁰ Linker molecules could be based on DNA strands,³⁷ which offer greater specificity in binding biomolecules tagged with complementary sequences. These scaffolding layers increase the effective thickness of the intervening hydrated layer between the membrane and the solid support, enabling the inclusion of transmembrane proteins such as ion channels or receptors that otherwise would be excluded from the bilayer.

In addition to bilayers, it is possible to form phospholipid monolayers on solid supports, which have traditionally been studied at the air-water interface of a Langmuir-Blodgett (LB) trough.^{38,39} Unlike lipid bilayers, which require high-energy, hydrophilic substrates on which to self-assemble, lipid monolayers can form on low-energy, hydrophobic surfaces such as SU-8 photoresist,⁴⁰ and alkanethiol self-assembled monolayers on gold,^{41,42} . Lipid monolayer formation and patterning have been demonstrated on graphene sheets.⁴³ Formation of “upside-down” monolayers on high-energy surfaces, featuring tight binding of lipid head-groups to the

substrate and hydrophobic tail-groups to the air, can be attained using the Langmuir-Blodgett method, or by depleting the mobile upper leaflet of a bilayer, leaving the bottom leaflet immobilized on the substrate.^{44, 45,46,47}

Several groups have tethered lipid vesicles to supported lipid bilayers using DNA hybridization^{48,49,50} or biotin-streptavidin recognition,^{51,52,53,54} enabling the organization of transmembrane proteins shielded from the solid support by the intervening supported lipid bilayer. Because tethered vesicles are anchored to a fluid membrane, they can diffuse on the surface and interact with other tethered vesicles to induce complex reactions.⁵⁵ Electric fields parallel to the substrate surface can be used to organize charged vesicles by electrophoresis or electro-osmosis.⁵⁶ This opens up the possibility of using tethered vesicles to detect analyte binding to ligand-gated ion channels by monitoring the net charge on the vesicle.⁵⁷

Linking molecular scaffolds can be patterned in a variety of ways to control membrane lateral organization further. Polymer-based dry lift-off,^{58,59,60} involving peeling off of a stencil layer from the membrane-supported substrate while the substrate is immersed in water, enables the same level of spatial control of lipid bilayers as conditional lift-off techniques, without harming the bilayer from traditionally-employed organic solvents used to remove the sacrificial layer. Microcontact stamping or posting involving the transfer of lipid bilayers from a microfabricated polymeric stamp such as PDMS to a supporting substrate allows for the sequential deposition of different lipid phases.^{61,62} This technique was used to create both positive and negative pattern transfers of bilayer microdomains, such as the patterning of fluid liquid-crystalline lipid patterns (DLPC, $T_m \sim -1^\circ \text{C}$) in a gel-phase lipid matrix (DPPC, $T_m \sim 41^\circ \text{C}$), or gel-phase islands in a sea of fluid lipids.⁶³ Scanning probe patterning methods have also been used to pattern sub-micron scale structures, such as lipid deposition from direct-write dip-

pen nanolithography,⁶⁴ or the creation of sub-micron voids scratched from a bilayer with an AFM tip.⁶⁵

The molecular-based scaffolds that have been described thus far share the common feature of an underlying solid inorganic substrate, such as silicon, metals or oxides, and can therefore be classified as two-dimensional. Even scaffolds consisting of polymer cushions or other spacer layers that decouple the lipid bilayer from the substrate can be considered to be effectively two-dimensional since the thicknesses of these layers (nm-scale) are generally orders of magnitude smaller than lateral scales (micron scale and higher), and membrane-scaffold molecular interactions still occur parallel to and immediately underneath the plane of the bilayer. Another approach that results in membrane-scaffold interactions that are truly three-dimensional involves electrospinning to form nanofibrous phospholipid and neutral lipid matrices that can condense into three-dimensional, high surface-area membranes.⁶⁶ The durability of such membranes can be enhanced with the sol-gel synthesis of polymerizable cholesteryl-succinyl silane (CSS), which forms extended, mechanically robust inorganic polysiloxane and silicate networks.⁶⁷ These membranes were functionalized with murine anti-CD20 antibodies, which enabled them to recognize and capture CD20-positive B-cell lymphoma cells, while CD20-negative Jurkat T cells did not bind specifically to the membrane. In this way, the electrospun fibrous membrane demonstrated the capability of functionally immobilizing antibodies and capturing specific cell types in a fibrous matrix consisting of polymerized and mechanically robust molecular linkages, just like a native cytoskeleton.

A variety of hydrogels have been used as flexible supports for planar bilayer membranes as well, including biological hydrogels, such as agarose, alginate and chitosan, and synthetic hydrogels, including PEG, polyacrylates and poly(vinyl alcohol) (PVA).⁶⁸ Biological hydrogels

suffer from lack of stability, while synthetic hydrogels typically require toxic solvents, initiators, crosslinking agents and unreacted monomers, limiting their use. It is possible, however, to form stable, synthetic PVA-based hydrogels with crosslinks consisting of a network of hydrogen bonds formed by carrying out repeated freeze-thaw cycles. By avoiding thermal or (photo) chemical initiators for crosslinking reactions, PVA hydrogels have been prepared under physiological conditions, including physiological pH.⁶⁹ These hydrogels can function as implantable tissues/organs, or drug delivery vehicles. Biomimetic sensing can be integrated in these hydrogels by loading them with liposomes containing reconstituted ion channels.

Solid state scaffolds

In addition to molecular-based scaffolds, the solid support itself can be modified through lithography and micromachining, involving combinations of material deposition, patterning, and etching. Lipid bilayers can span these micro- and nanoscopic features, or conformally coat them, depending on a number of factors including feature size, solution pH, the extent of screening of electrical charge as a function of solution ionic strength,⁷⁰ or the method used to form the bilayer, such as vesicle rupture, film transfers using Langmuir-Blodgett or Langmuir-Schaefer techniques, or bubble collapse deposition.^{71,72} Spreading of lipid bilayers on solid supports has also been controlled by variation of surface energy, for example, by patterning hydrophobic areas in a hydrophilic background, or hydrophilic areas in a hydrophobic background. In addition to providing a supporting scaffold for the membrane, metallic features enable electrical recordings of ion channel activity, as well as innate membrane capacitance and conductance.

To investigate the importance of substrate smoothness, self-spreading of lipid bilayers was studied on nanostructured silicon surfaces featuring 100 nm grooves, where it was found that the spreading patterns tended to be elliptical in shape, instead of the having the usual circular

shape seen on smooth surfaces.⁷³ A balance between the bending energy cost needed for the bilayer to conform to the corrugated surface and the adhesion energy gain determined the extent of lipid bilayer spreading, as well as film stability. Arrays of nanoscale obstacles have been fabricated on supporting substrates to study how the lateral flow of fluid lipid molecules in the supported lipid bilayer membrane was perturbed by pinning centers.⁷⁴ The evolution of the membrane interface was found to obey a deterministic growth model at short times, but not at longer timescales, corresponding to a slowing down of the membrane spreading rate with time.

Starting with simple scratches on silica surfaces, a number of lithographically-defined cytoskeletal barriers have been engineered at micron and nanometer length scales on substrate supports to restrict bilayer diffusion, including metallic, polymeric and immobilized lipid or protein barriers.⁷⁵ These barriers form corrals that restrict diffusion between adjacent bilayer patches, while allowing free diffusion within each patch, resulting in two different time scales for diffusion, a short-range diffusion coefficient corresponding to free diffusion within the corrals, and a longer time constant for diffusion across barriers, similarly to behavior that has been observed with single-particle tracking measurements of proteins and lipids on live cell surfaces, where membrane molecules were observed to undergo random, Brownian movement on small length scales, but were confined most of the time to patches on the cell surface.⁷⁶

Colloidal lithography based approaches have been used as tunable filters for different lipid molecules. Discontinuous metal structures behaved as two-dimensional porous films that also hindered lipid motion, resulting in a tractable model for subdiffusion in bilayer films, described by $x^2 = 2dDt^\alpha$ with $\alpha < 1$.⁷⁷ Similar behavior was seen with fluorescence correlation spectroscopy (FCS) where avidin bound to a surface-supported lipid bilayer exhibited anomalous subdiffusion at concentrations in the bilayer above about 5%.¹²

Sub-micrometer length scales, accessible through electron beam lithography (EBL), are oftentimes necessary for mimicking membrane-cytoskeletal interactions. Arrays of corrals patterned on a supporting substrate create barriers to diffusion (at length scales of hundreds of nm), which can alter downstream signaling processes within a cell. For example, when T cells interacted with a supported lipid bilayer to which T Cell Receptor (TCR) complexes had been tethered, nanometer scale metallic barriers that emulated membrane-cytoskeletal interactions by dividing the cell-substrate interface area into sub-regions resulted in enhanced downstream signaling from TCR microclusters that had been mechanically trapped at the periphery of the interface (figure 3).⁷⁸ In later work, it was seen that lithographically defined lipid bilayers containing arrays of short, linear barriers blocked the migration of TCR clusters from the periphery of the immunological synapse in toward the center at early times, but that the clusters were routed around the edges of the linear barriers rather than crossing the barriers.⁷⁹

Real cytoskeletal barriers to diffusion are often transient or semipermeable to diffusion of lipids or proteins, which can be modeled by inserting staggered gaps at specific locations along a metallic diffusion barrier. Using EBL, arrays of linear chromium barriers 30 nm in width were fabricated, interspersed with gaps that resulted in unhindered diffusion parallel to the barriers and blocked movement across them. This resulted in anisotropic diffusion, which was characterized with Fluorescence Recovery after Photobleaching (FRAP) measurements.⁸⁰

Thus far, membrane-cytoskeletal models have been described that employ modifications of supported lipid bilayers on flat surfaces. However, cell membranes are not flat. This issue is being addressed by many groups by investigating lateral diffusion in lipid bilayers supported on high-curvature micro/nanoporous silica substrates.^{81,82} Spherically supported bilayer lipid (SSBL) membranes consist of silica beads ranging in diameter from about 50 nm to about 80

μm , which support a fluid lipid bilayer membrane in the same manner as traditional supported lipid bilayer membranes on flat hydrophilic substrates, but on supports with radii of curvature in the size range from tens of nanometers, corresponding to small unilamellar vesicles (SUVs), to tens of micrometers, corresponding to cells and giant unilamellar vesicles (GUVs).⁸³ SSBL beads have been used to introduce model lipid membranes to cells, and to organize the components of real cytoskeletons when co-cultured with cells. For example, when SSBL-beads were allowed to interact with rat embryonic hippocampal neurons for 24 hours, it was found that actin filaments and microtubules preferentially assembled in the vicinity of the fluid lipid domains on the SSBL beads, not the gel-like lipid domains.⁸⁴

Micro- and nanofabrication of well-defined cytoskeletal mimics with controlled topography have produced further insights, by capturing more quantitative information that describe how surface roughness and curvature impact the organization and localization of lipid bilayer components in greater detail. For example, mixed regions of nanoporous and smooth textures, fabricated with microscale patterning via photolithography on the same substrate, have revealed subtle yet important differences in lipid mobility and organization in supported lipid membranes caused by spatial variations in surface roughness and adhesion.⁸⁵ Hybrid micro/nanostructured supported lipid bilayers have been created by the self-assembly of high density lipoproteins that form nanoscale discs that corral lipids, which can themselves be patterned on a supporting substrate at the microscale using short-wavelength UV photolysis through a physical shadow mask. In another study, fluid POPC bilayers were observed to conform to the sides of grooved silicon gratings a few hundred nm deep. It was found that quantitative analysis of anisotropic diffusion along the channel length versus width at the *microscale* was sufficient to yield information about the *nanoscale* morphology and dynamics.⁸⁶

In studies of lipid-cholesterol mixtures in double bilayer assemblies on supports with a high density of stripes, patterned with interferometric lithography to have microscale widths, but nanoscale heights, phase separation was observed to be strongly linked to the local degree of curvature.⁸⁷ Another study employing interferometric lithography to create nanotextured undulations in silicon with radii of curvature ~ 175 nm used mixtures of short and long chain length phosphatidylcholine lipids to form planar assemblies with variable thicknesses. AFM and quantitative confocal fluorescence microscopy were used to verify that the films formed both supported and suspended bilayers, in registry with the crests and troughs of the nanoscale channels on the substrate.⁸⁸

In addition to interactions with micro/nanofabricated scaffolds that form corrals in the membrane, demixing involving phase separation of binary and ternary mixtures of lipids in the membrane itself can also induce lateral organization with the formation of microdomains.⁸⁹ Lateral diffusion is hindered by microdomains differently than in corrals formed by synthetic scaffolds, within which molecular motion remains essentially unhindered. For both cases, diffusion coefficients decrease with increasing observation time, but in the case of corrals, long-range diffusion can be described by a “hopping” diffusion between adjacent corrals that is different than the more complex long-range diffusive behavior of molecules in the presence of microdomains, which depends on the size, shape, and distribution of gel-phase obstacles in the membrane.⁹⁰ For example, it has been observed from FRAP and AFM measurements of demixed DLPC/DSPC supported lipid bilayers on mica that lateral diffusion of molecules in the fluid phase was obstructed by the formation of stochastically distributed gel-phase microdomains in the membrane, and was dependent on the size, shape, and area fraction of the domains, in accordance with percolation theory.⁸⁹

Alternatively, the underlying supporting scaffold can be used to spatially control the distribution of microdomains in the membrane, thereby controlling the diffusion of molecules in more subtle ways than by a scaffold directly organizing fluidic lipids into corrals. It was found that a lipid bilayer comprised of a binary mixture of gel and fluid phase lipids (DOPC/DSPC) followed the contours of a silica xerogel surface comprised of ~ 50 nm diameter porous silica beads, and that the corrugation of this surface resulted in a lipid density in the fluid regions that was twice that on a smooth mica surface, while the gel-phase lipid domains were larger in size and less numerous.⁹¹ In addition, with both FRAP and FCS, the lateral diffusion coefficient in the fluid regions on the xerogel surface was observed to be half that on mica, consistent with the picture of a bilayer closely following the surface roughness. In a more complicated ternary mixture comprised of DOPC/DSPC lipids and cholesterol, the coexistence region of the phase diagram of the mixture stretched toward increased cholesterol concentrations for the higher-curvature xerogel surface compared to mica, suggesting that the higher curvature resulted in lipid demixing.⁹² More precise control of the formation of liquid-ordered phase domains was obtained by top-down fabrication of a scaffold consisting of topographic walls and nanocorrugated topographical features. The liquid-ordered phase lipids became confined between elastic energy barriers present at the edges of the topographical features as a consequence of the larger bending rigidity resulting from the more tightly packed hydrocarbon chains.⁹³

Micro/nanoporous arrays

Incorporation of ion channels into synthetic suspended or supported bilayers has been and remains one of the most compelling reasons for creating and studying bilayer-supporting structures, particularly for pharmaceutical drug discovery and medicine.^{18,94} One of the first synthetic bilayer systems that was developed to study reconstituted ion channels was the “black

lipid bilayer” (BLM), which involved the formation of a free-standing lipid bilayer across an aperture a few hundred microns in diameter in a thin partition in aqueous solution, either by “painting” lipid molecules dissolved in an organic solvent across the aperture (Müller-Rudin technique), or by using the Langmuir-Blodgett technique, which did not require the use of volatile organic solvents, but was cumbersome to implement.^{18,22} It was recognized that BLMs enabled more precise control of membrane composition than other techniques for ion channel recording like patch clamp measurements of cells, but there were also difficulties, including residual solvent left in the bilayer, the fact that LB membranes were prone to defects, and short lifetimes (up to only a few hours), which prevented extended monitoring of ion channels. Attempts to increase the stability and reproducibility of BLMs included systematically testing different materials having varying degrees of surface energy and charge such as Teflon,⁹⁵ plastics,⁹⁶ and glass,⁹⁷ and by chemical functionalization of surfaces.⁹⁸

Lipid bilayer mechanical stability and lifetimes were significantly enhanced, however, when suspended by pores fabricated at nanometer scales, for example, by focused ion beam milling or electron beam lithography on silicon nitride membranes,⁹⁹ or by using nanoporous alumina substrates.^{100,101} Free-standing nanopore-spanning bilayers have been assembled on nanoporous membranes using the lipid painting technique,¹⁰² or solvent-free approaches, such as deposition of lipids from cell membranes,¹⁰³ monolayer-coated bubbles,⁷¹ or by vesicle fusion.¹⁰⁴

“NanoBLMs” created using the lipid bilayer painting technique seemed to follow a similar “zipper” formation mechanism to traditional larger-scale BLMs and solid-supported membranes. In this mechanism, thinning of the suspended membrane from the initial thickness of several μm to about 100 nm occurred as solvent drained away, induced by random thermal or mechanical motions and driven by the Laplace hydrostatic pressure difference at the border of

the flat film and the solvent annulus, known as the Plateau-Gibbs border.¹⁰⁵ The final bilayer thickness of a few nm was determined from two opposing forces, the London-van der Waals attractive force between the separated aqueous phases on either side of the bilayer, and steric repulsion of the apposed lipid acyl chains.¹⁶ It was found for the larger-scale apertures that formation of a stable bilayer required a minimum initial surface area in order to avoid separation of existing bilayer regions by the solvent due to Marangoni flows, behavior that was seen for SLBs on continuous solid supports as well.¹⁰⁵ Because the nanoBLMs could span multiple nanopores separated from each other by solid-supported areas, the destabilizing effect of a Plateau-Gibbs border at any one given nanopore was not as significant. However, just as with larger traditional BLMs, specific capacitances were low, an indication that the nanoBLMs contained residual trapped solvent, which can be problematic, since the solvent can denature proteins, and destabilize the bilayer as it evaporates.^{106,107}

A better understanding of how nanopore-spanning lipid bilayers form for the solvent-free vesicle fusion technique was obtained by statistical analyses of several thousands of individual nanopores obtained with high resolution laser scanning confocal fluorescence microscopy,¹⁰⁸ in conjunction with ensemble measurements such as energy dissipation monitored with a quartz crystal microbalance and FRAP. It was found that applying vesicles to the substrate with diameters larger than those of the nanopores was necessary to avoid conformal bilayers. This is consistent with other examples that have been published in the literature of small unilamellar vesicles (SUVs) forming bilayers that span 100 nm diameter pores,¹⁰⁰ and GUVs forming bilayers that can span pores an order of magnitude or so larger.¹⁰⁹ However, controlling the vesicle size relative to the pore diameter was, by itself, not necessarily a sufficient condition for successfully forming a nanopore-spanning bilayer membrane. Whether or not a lipid bilayer

spans a nanopore opening or conformally coats the pore interior depends on the magnitude of the bending energy cost required for a bilayer to deform and closely follow the curvature of the pore rim relative to the adhesion energy.¹⁰⁸ For a strongly adhering bilayer, the bending energy penalty for deforming the bilayer will be less than the adhesion energy, and the bilayer will conform to the surface. Generally, low values of the adhesion energy and pores with high enough curvature, particularly at the pore rim, will result in greater probability for forming nanopore-spanning membranes across apertures.

The elastic response of nanopore-spanning lipid bilayers, from both solvent-bearing nanoBLMs, and solvent-free nanopore-spanning bilayers formed from vesicles, was determined from force indentation experiments carried out with an AFM tip in the center of a number of membrane-covered pores.¹¹⁰ The elastic response upon indentation for both types of nanopore-spanning membranes was linear, allowing the assignment of an apparent spring constant for each type of membrane. However, there were also significant differences (figure 4). For the nanoBLMs, the free energy difference between the membrane at the pore rim, which consisted of the upper leaflet of the bilayer on top of a chemisorbed hydrophobic sublayer, and the free-standing bilayer over the center of the pore led to a substantial pre-stress in the bilayer due to lateral tension, which resulted in spontaneous rupture of the membrane at very low area dilation (less than 2%). This was in addition to the destabilizing effects of the residual entrapped solvent in the membrane. The energy penalty for bending the membrane over this rim edge was significant relative to the lateral tension for pore diameters less than about 40 nm.¹¹⁰ In solvent-free membranes, on the other hand, the lateral tension was significantly reduced compared to nanoBLMs because there were no substantive changes in composition or structure of the bilayer from the rim to the center of the pore. Membrane rupture was not observed on indentation, even

for area expansions greater than 5%. In this case, both lateral tension and bending energy contribute to the elastic response upon indentation.¹¹⁰

The mechanical properties of free-standing lipid bilayers on nanoporous Si₃N₄ supports could be stabilized by the membrane-associating protein Annexin-A5, which assembles on lipid monolayers and bilayers containing negatively-charged lipids in a reversible Ca²⁺ dependent manner.¹¹¹ Proteolipid monolayers and bilayers were formed at the air-water interface of a Langmuir trough, and transferred to the nitride supports as vertical Langmuir-Blodgett (LB) films or horizontal Langmuir-Schaeffer (LS) films. Choice of surface functionalization on the nitride support, for example, rendering the surface hydrophobic versus hydrophilic, affected how the membrane associated with the pore edges, thereby fundamentally changing the manner in which the Annexin A5 proteins assembled on the membrane. A hydrophobic nitride surface resulted in transfer of a lipid monolayer to the substrate and stabilization of the monolayer across the pore through association of the lipid head groups with Annexin-A5, mediated by Ca²⁺.¹¹¹

The ability to reconstitute ion channels and other membrane proteins in nanopore and micropore-spanning lipid bilayers has enabled new classes of membrane protein assays for biosensing and drug targets.¹⁴ A critically important feature of artificial membrane systems in this regard is the ability to characterize many different proteins with single-molecule resolution. Electrophysiology is the measurement of the electrical properties of membranes, both native and artificial, including the capacitance, resistivity, and polarizability of membranes and the transport of ions across the membrane controlled by transmembrane proteins. These measurements provide information on the material and structural properties of the membrane, such as membrane area and dielectric constant. High electrical resistance levels across an intact membrane (~10 GΩ) increase the sensitivity for detecting individual channel currents at the

single-molecule level. These levels are on the order of a few pA, and are well within the range of commercial trans-impedance amplifiers.

Procedures for incorporating ion channels or other membrane proteins into bilayers either suspended over micro or nanopores or supported on a surface include insertion of membrane proteins solubilized with detergent,^{112,113} fusion of proteoliposomes,^{114,115} or transfers from cells, either as direct transfers from the cell wall,¹¹⁶ or the transfer of native proteins from proteovesicles from lysed cells.¹¹⁷ Several groups have demonstrated the ability to insert and measure single-channel recordings in nanoBLM membranes from channel-active peptides like gramicidin, alamethicin⁹⁹ and valinomycin,¹¹⁸ the α -hemolysin pore from *Staphylococcus aureus*,¹¹⁹ the proton pump bacteriorhodopsin,¹²⁰ the F₀F₁-ATPase motor¹²¹ and the large outer membrane protein OmpF from *E coli*.¹⁰² A possible complicating factor with nanoBLM membranes, as with black lipid membranes in general, is the presence of residual solvent in the bilayer, as discussed earlier. Other groups have demonstrated the ability to insert peptides and proteins into pore-spanning bilayers that are completely free of solvent, using vesicle fusion for example. To achieve a high enough electrical resistance needed to detect single ion channels in the membrane, the substrate surface was functionalized to be hydrophobic to prevent the formation of an aqueous layer between the bilayer and the support, which could serve as a reservoir for the conduction of ions.¹²² The most useful geometry for these assays was one where the proteins were integrated in the membrane only over the pores to prevent interactions with the supporting surface, and both sides of the bilayer were accessible to aqueous solution. Fluorescence and electrical measurements could be used to verify that the bilayer spanning each pore was fluid, and that the channel-forming molecules were located only in the pore areas of the substrate.¹⁰⁸

Thus far, single-channel devices have been described that use thin nanoporous or microporous Si_3N_4 membranes to prepare stable, free-standing lipid bilayers, for both nanoBLMs and the solvent-free vesicle fusion technique. An important challenge that needs to be overcome is the high capacitance of the ultrathin silicon nitride membrane, which can impede voltage-clamp detection of single ion channels.⁹⁹ Devices fabricated with materials other than silicon/silicon nitride offer alternatives with tunable electrical properties. Examples include anodized alumina, porous polycarbonate, polystyrene and PET membranes, photoresist, and glass nanopores.

“Smart” polymer brush nanostructures have been developed that result in the controlled immobilization of membrane proteins in lipid bilayers suspended over nanopores.¹²³ The inner Si_3N_4 walls of the nanopores in the array on the *trans* side of the membrane were each functionalized with pH-sensitive poly(methacrylic acid) (PMAA) brushes via surface-initiated atom transfer radical polymerization of sodium methacrylate. Nitrilotriacetate (NTA) pendant groups were attached through amide coupling chemistry to activated carboxylate groups on the polymer. This strategy combined the ability to reversibly open or close the nanopores with changes in pH with the ability to direct the capture of membrane-bound His-tagged proteins (in the presence of Ni^{2+} ions) only at the nanopores.¹²³

Integration of micro/nanofabrication and microfluidics

The combination of microfabrication and microfluidics has made it possible to reconstitute and study membranes and membrane-bound proteins on a massively parallel scale, and has resulted in the ability to ask and answer fundamental questions about membrane biophysics that had remained elusive.¹⁴ Microfluidic platforms have the capacity to precisely control sub-microliter volumes of reactants in high-throughput experiments, at faster reaction

times, and with the ability to separate and control individual chemical and/or physical reaction steps of complex systems in time and space by controlling liquid flow in microchannels.¹²⁴ This level of spatiotemporal control presents opportunities to create and investigate increased structural and functional complexity of membrane systems, by controlling the timing, location and interconnectivity of elementary reaction steps. Microfluidic lab-on-a-chip platforms operate at cellular length scales, and provide for simultaneous electrical and optical characterization with embedded microelectrodes and optical elements ranging in complexity from optically transparent materials to integrated plasmonic structures for label-free detection of biomolecules.¹²⁵

Recently, microfabricated devices have been developed that feature lipid bilayer membranes formed simultaneously in hundreds to thousands of microchamber arrays.¹²⁶ The microchamber membrane arrays correspond to femtoliter (10^{-15} L) volumes, and were formed *in situ* in a microfluidic device featuring arrays of hydrophilic patches in a hydrophobic background. Because the membranes could be formed from a pre-programmed sequence of injection steps in the microfluidic device, asymmetric bilayers, i.e., bilayers where the compositions in the top and bottom leaflet were different, could be assembled in a high throughput manner, which would be difficult for traditional methods that rely on spontaneous assembly of lipid membranes.¹²⁶ Asymmetric membranes were formed spanning the top of each hydrophilic array site after sequential injections of liquids into the channel, in a technique similar to the “painting” of lipid bilayers in nanoBLMs. Bilayer compositional asymmetry was demonstrated by creating a pH step across the membrane, monitored with the pH-dependent emission of fluorescein tags located on the bottom leaflet, which could be quenched only by the insertion of α -hemolysin pores in the membrane. Lipid flip-flop events between leaflets were monitored with the reduction and subsequent loss of fluorescence of nitrobenzoxadiazole (NBD)

tags, located originally only on the bottom leaflet, by the action of dithionite molecules located on the opposite side of the bilayer¹²⁶

More recently, 10^5 of these femtoliter-volume microchambers were used by the same group to characterize stochastic and quantized ion transport from single molecules of α -hemolysin, for passive transport, and F_0F_1 -ATP synthase, for activated transport.¹²⁷ Lipid bilayers formed in each chamber were stable against permeation by fluorophores or ions, and the ultra-low detection volume improved sensitivity. For the α -hemolysin assay, assuming the nanopore opening of the protein was the rate-limiting step for ion transport across the membrane, and applying Fick's law, led to the determination of the diffusion coefficient of a fluorophore in the protein pore that was about an order of magnitude less than the diffusion coefficient of the fluorophore in aqueous solution. For the F_0F_1 -ATP synthase assay, it was determined that the proton-pumping rate of a single molecule of F_0F_1 -ATP synthase was 27.5 protons/second, corresponding to an ATP hydrolysis rate of 8.4 turnovers per second, which was about 36x slower than in detergent-solubilized F_0F_1 -ATP synthase.¹²⁷ These differences were probably related to the more viscous environment of the membrane compared to aqueous solution. Future work with this platform will target receptor-mediated signal transduction, membrane fusion or fission, and confinement of cell-free gene transcription and translation, genome replication and signaling cascades.^{127,128}

Other groups have modified the procedure for forming bilayer membranes *in situ* in microchannels by using water vapor-saturated air as one of the injectable fluids. This resulted in extended arrays of supported bilayers that each conformally adhered to the bottom of the wells through multiphase microfluidic engraving,¹²⁹ in contrast to bilayers that span wells. These supported lipid bilayer arrays retained their natural two-dimensional fluidity and phase behavior,

and provided a protective, biologically compatible barrier that prevented the cells from irreversibly adhering to the bottom of the wells. As a proof of principle, human red blood cells that were trapped in the microfluidically engraved array sites and exposed to 1% ethanol solution exhibited structural changes from normal morphology to highly spiculated shapes over time.

It has also been shown that shearing forces from bulk flows in a microfluidic device can drive a bilayer, initially formed by vesicle rupture, over a flat hydrophilic SiO₂ surface along the floor of the channel to a region containing submicron embedded wells. Migration of the bilayer from a flat region to the nanowell region after it had already been formed relaxed the constraint of finding interactions between the lipid bilayer and the surface that are strong enough to result in vesicle adsorption and rupture, and provided additional parameters of the bulk solution for optimizing bilayer formation and spreading that can be systematically varied, including salt content and pH.¹³⁰ Interestingly, a bulk solution pH of 8.0 resulted in the bilayer conforming to the sides and bottoms of 80 nm diameter wells while a pH of 9.5 resulted in the bilayer spanning the wells.

Droplet-based lipid bilayers at interfaces

Bilayer membranes have also been formed at oil-water interfaces in emulsions and droplets as an alternative approach for development of synthetic model membrane systems. Taking advantage of the tendency for lipid bilayers to form spontaneously at the interface between immiscible phases, emulsion-based or droplet-based interface bilayers (DIBs) have been created on demand by bringing two or more aqueous droplets dispersed in oil together.^{131,132} Aqueous droplets functionalized at the water/oil interface with monolayers of lipid molecules spontaneously form lipid bilayers when two or more droplets make contact. DIB systems, ranging in complexity from a single bilayer membrane at the interface between two aqueous

droplets to extended droplet-based networks, have been created and manipulated in a number of ways, including manual assembly using micromanipulators,¹³¹ creation in microfluidic platforms,¹³² including droplet microfluidics,^{133,134} optical tweezers,¹³¹ and the use of electrowetting on dielectrics (EWOD) or dielectrophoresis to control the movement of droplets on substrates with electric fields.¹³⁵

One attractive feature of DIBs systems is the ease in incorporating microelectrodes in the droplets for controlling droplet movement, or for capacitance and electrophysiological measurements. However, the membranes are typically vertically oriented, making optical imaging of them difficult. In order to be able to simultaneously image bilayers with a microscope while measuring electrical signals, “hybrid” droplet/hydrogel interfaces have been developed that position the horizontally oriented bilayer interface within the working distance of high numerical aperture objectives for epifluorescence, confocal microscopy, or within the evanescent optical field for total internal reflection fluorescence (TIRF).¹³⁶ Incorporation of microelectrodes on either side of the interface allows the simultaneous recording of optical signals and channel currents with voltage clamp methods or with electrochemical impedance spectroscopy. The hydrogel serves as both a flexible support for the bilayer and as a conduit for ionic currents.

Nanostructured surfaces impregnated with hydrogel could serve as synthetic “cytoskeletal” scaffolds that support and interact with the bilayer at the hybrid droplet-gel interface in much the same way as a real cytoskeleton does with a cell membrane, for example, by imparting well-defined lateral structure and distributed substrate attachments to the bilayer that will emulate the connections of cellular membranes to the underlying cytoskeleton. We have recently developed methods for forming bilayer interfaces between two or more aqueous droplets on oil-impregnated nanostructured surfaces in air (“airDIBs”).¹³⁷ Capillary action resulted in a

very thin layer of oil coating the droplets in air that prevented their coalescence. Incorporating phospholipid molecules in the aqueous droplets resulted in single-channel electrical behavior similar to what has been seen in other bilayer systems, but as far as we know, this represents the first demonstration of suspended lipid bilayers that have been created and studied in an ambient environment rather than submerged in a fluid reservoir. This also opens up the possibility for sensing of airborne molecules with airDIBs from molecules that diffuse or migrate to the bilayer after entering the droplet or gel from the ambient environment.

Future Studies

Nanofabricated scaffolds interacting with lipid bilayer membranes are useful models for understanding the interactions between the cell membrane and the cytoskeleton, including how these interactions influence cellular signaling. The main contributions these synthetic model systems have made so far are elucidating how the nanostructured environment regulates molecular diffusivity in the membrane, both locally and long range. Future studies will uncover more detailed information on localized domains induced by membrane-cytoskeletal interactions (“lipid rafts”) and how these impact signaling. Neutron reflectometry studies¹³⁸ will play an important role in determining the structure of the scaffold-membrane interface at high resolution. Fitting scattering length density profiles to the reflectometry data will provide a detailed view of structure normal to the surface for the membrane and its interface with surrounding water and silicon. Neutrons are superior to x-rays in this regard, as they can readily distinguish lipid tails from water and phosphorus from silicon. Previously, we used neutron reflectometry to examine the structure of lipid bilayers deposited on two-tier substrates possessing both microscale and nanoscale features and were able to differentiate the lipid bilayer material trapped within nanoscopic roughness from the bilayer that remained freely suspended over the roughened

features.¹³⁹ These studies will be complemented by both single-molecule and ensemble quantitative fluorescent techniques for determining lipid domain formation and lateral intramembrane molecular transport.^{140,141,142,143,144}

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Figure 1. Examples of nanostructured scaffolds that can mimic the cytoskeleton, including nanofibrous structures (Ref. 67), deterministically patterned nanopillars with EBL (Ref. 26) and stochastically patterned nanopillars (Ref 137) and nanopores (Ref.101).

Figure 2. Different arrangements of supported lipid bilayer membranes. (Left) Electrical measurements of transmembrane ion channels in membrane tethered to gold electrode with thiolipids. (Right) Membrane detached from live cell supported by silica microbead. Adapted from Ref. 159.

Figure 3. Bilayers containing arrays of short, linear barriers blocked the migration of T-cell receptor (TCR) clusters from the periphery of the immunological synapse in toward the center, resulting in enhanced signaling from the mechanically trapped TCR microclusters. Adapted from Ref. 78.

Figure 4. Structural differences between (a) nanoscale black lipid membrane (nanoBLM) spanning gold-coated pore rim functionalized with hydrophobic octanethiol monolayer, resulting in significant pre-stress and residual solvent trapped within the bilayer and (b) solvent-free lipid bilayer spanning hydrophilic pore exhibiting negligible pre-stress. Adapted from Ref. 107.

Figure 5. “Hybrid” droplet/hydrogel interfaces have been developed that position the horizontally oriented bilayer interface within the working distance of high numerical aperture objectives for TIRF. Incorporation of microelectrodes on either side of the interface allows the simultaneous recording of optical signals and channel currents. Adapted from Ref. 136.