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Review

Templating membrane assembly, structure, and dynamics using engineered interfaces

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ABSTRACT

The physical and chemical properties of biological membranes are intimately linked to their bounding aqueous interfaces. Supported phospholipid bilayers, obtained by surface-assisted rupture, fusion, and spreading of vesicular microphases, offer a unique opportunity, because engineering the substrate allows manipulation of one of the two bilayer interfaces as well. Here, we review a collection of recent efforts, which illustrates deliberate substrate–membrane coupling using structured surfaces exhibiting chemical and topographic patterns. Vesicle fusion on chemically patterned substrates results in co-existing lipid phases, which reflect the underlying pattern of surface energy and wettability. These co-existing bilayer/monolayer morphologies are useful both for fundamental biophysical studies (e.g., studies of membrane asymmetry) as well as for applied work, such as synthesizing large-scale arrays of bilayers or living cells. The use of patterned, static surfaces provides new models to design complex membrane topographies and curvatures. Dynamic switchable-topography surfaces and sacrificial trehalose based-substrates reveal abilities to dynamically introduce membrane curvature and change the nature of the membrane–substrate interface. Taken together, these studies illustrate the importance of controlling interfaces in devising model membrane platforms for fundamental biophysical studies and bioanalytical devices.

Contents

1. Introduction

Bimolecular organization of lipids, or the lipid bilayer configuration, represents a universal motif for controlling cellular organization in living systems [1]. It represents a defining feature of biological membranes [2], and generally serves to compartmentalize cellular functions, as well as mediate many biological functions by providing a defined surface/interface for cell-surface recognition, signaling, and transport [3]. From a structural point of view, a unique feature of the lipid bilayer is that it is bounded by two independent aqueous phases, e.g., intracellular milieu and the extra-cellular matrix for plasma membranes, which in turn produces two topologically connected membrane–water interfaces separated only by a barrier of 4–5 nm [4]. Precise physical properties of each of these two, bilayer–bulk water and bilayer–substrate, interfaces, and the structural and compositional gradients they generate, influence the organization and many physical and chemical properties of biological membranes.

Over the past decades, understanding the importance of membrane interfaces in modulating membrane physical and chemical properties has been greatly facilitated by model membrane configurations. The earliest model systems for biological membranes were the so-called "black lipid membranes," which consist of a phospholipid bilayer across an aperture separating two independently adjusted semi-infinite aqueous compartments [5–7]. Closed model

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membranes, including uni- and multilamellar phospholipid vesicles, known as smectic mesophases [8] or liposomes [9], have afforded studies of how lipid organization is influenced by single compartmentalized interfaces embedded in semi-infinite aqueous ambient phases. Further, the organization of lipids as single monomolecular "Langmuir" layers at the aqueous interfaces of air [3,10–12] represents another generic class of model membrane configurations that has yielded useful information regarding the role of single membrane–water interfaces in modulating properties of the lipid layer. But while the interfaces of lipid with its surroundings are important in defining the physical properties of lipid films in any configuration, supporting lipid bilayers on solid surfaces allows the unique opportunity to manipulate its interfaces, for instance by engineering the substrate, thus controlling the physical properties in a defined manner.

From this vantage point, a systematic control of the property of specific membrane interfaces can best be achieved using various solid supports to template lipid organization at their surfaces. These so-called supported membranes [13] can be formed simply by presenting vesicular microphases of lipids to the solid–liquid interfaces. Under a broad range of ambient conditions, the vesicles rupture and spread spontaneously at the solid templates producing well-defined membrane phases [14]. Alternatively, they can also be formed from dried lipid stacks, which spread upon controlled hydration over the template surface [15,16]. The macroscopic character of the templating support is "epitaxially" reflected in the membrane organization. For instance, planar, tubular, and spherical substrates immediately impose respective geometrical constraints on the macroscopic nature of the membrane organization achieved. Furthermore, these substrate-supported bilayers are separated from the substrate surface through an intervening cushion layer [17] (e.g., a hydration layer of water variously estimated between 6 and 20 Å thick [18–20]), in equilibrium with its bulk surrounding on the opposite side of the bilayer.

Although water is present on both sides of the bilayer, the molecularly small thickness of the water-filled cleft between the substrate and the bilayer raises the question of whether the water in such narrow confinement can recapitulate the properties of the bulk water on the other side [21]. Further, in topologically or chemically structured surfaces, the amount of water at the substrate interface is unknown and the membrane–substrate interactions are likely altered [22]. In addition, the charge at the substrate surface has a strong influence on the overlying bilayer such as shown recently by us [23] and others [24]. While the physical properties of the substrate-bound hydration layer at the membrane–substrate interface remain incompletely understood [21,25], work in other systems can provide a rough qualitative estimate for how water can become structured when confined to ultra-thin layers (e.g., a thickness of only a few bilayers of water molecules). Several previous theoretical and experimental studies of water in confinement suggest that, in comparison to bulk water, water in ultra-thin layers is more ordered with higher viscosity and lower dielectric constants [26,27]. This picture of water confined between membrane and substrate surface argues against a complete decoupling of the membrane bilayer from substrate interactions. Consistent with these predictions, many examples now confirm that characteristics of the lipid bilayer remain at least partially coupled with many physical properties (e.g., charge, wettability, and topography) of the substrate surface.

Such membrane–substrate coupling can lead to important and often undesirable consequences. For instance, the presence of net substrate charge can significantly alter lipid assembly at the substrate surfaces inducing overall and leaflet-dependent compositional asymmetry in supported bilayers not found in their parent vesicles [23,24,28]. Similarly, a strongly interacting substrate can induce frictional coupling of at least the proximal (near-substrate) leaflet of the bilayer, which in turn may affect the leaflet–leaflet interactions [29–31]. Some recent studies also suggest independent melting characteristics for the proximal and the distal leaflets of bilayers when they cross their gel–fluid transition temperatures [32,33].

In this regard, many efforts have reported strategies to efficiently decouple substrate–membrane interactions. A central driving force for this focus is that the native hydration layer is insufficient in thickness to eliminate contacts between membrane proteins with large extra-membrane domains and the substrate. Such contacts may frustrate the conformational and translational dynamics required to reconstitute proteins in their functional states within the supported membrane configuration [13,34–36]. Moreover as noted above, there is also an appreciation that frictional or electrostatic coupling between the substrate and at least the proximal leaflet of the lipid bilayer may introduce undesirable asymmetries in structural, compositional, mechanical (e.g., drag), and dynamic properties of supported membranes. To this end, a variety of approaches aimed at cushioning the membrane–substrate interphase region using intermediate or intercalated "soft" cushioning layers of water (in the 10–100 nm thickness range) by incorporating hydrophilic tethers, hydrogels, polymers (e.g., poly(ethylene) glycol) and biopolymers (e.g., chitosan), all have proved successful [35,37–39,13,34,35,37,40–44]. These investigations have been recently reviewed elsewhere [13,35].

The notion that deliberate coupling of substrate properties with a membrane bilayer can produce potentially useful templated membrane configurations has, in contrast, received much less attention [45]. Because surface properties of solids, which include wettability, topography, dimensionality, and surface electrostatics, can all be systematically tailored, and in some instances dynamically altered, it appears that careful control of substrate properties can provide a simple and effective means to template many membrane properties including spatial molecular distributions, compositional heterogeneities, lateral tension, packing density, curvature, and even membrane morphologies. Moreover, recent advances in patterning and surface modification methods [46,47] (chemical and topographical) allow fabricating substrate surfaces that exhibit an unprecedented level of control of spatial variations in substrate properties at micro- to nanometer length scales. Use of such structured surfaces then provides a means to *spatially* vary substrate–membrane interactions, which in turn should template co-existing surface patterns of membrane properties within single lipid films. These opportunities are only beginning to be explored [48–54].

There are at least three additional reasons for developing the supported lipid bilayer (SLB) configuration. *First*, because of their thin-film format, SLBs are amenable to characterization by a broad variety of surface science based tools including most notably epifluorescence microscopy-based methods, scanning probe microscopies, and imaging ellipsometry. Indeed, the physical chemical characteristics of bilayer membranes have been greatly elucidated using the SLB construct. Examples include two-dimensional fluidity [55,56], material elasticity [57], electrical properties [58,59], phase behavior, including lateral phase separation [60,61], and fluid–fluid immiscibility (e.g., co-existing phases and lipid rafts) [62–65] *Second*, one of the original motivations for the development of supported membranes, namely presentation of membrane receptors to cells [66], is proving highly valuable in dissecting molecular events surrounding many classes of cell–cell interactions (e.g., immunological synapse, cell adhesion, inflammation, etc.) as well as in understanding how physical properties of membranes can be used to reprogram cellular behavior [67]. *Third*, because supported membranes integrate a fluid phospholipid structure with a solid surface, they are also relevant to the design of synthetic biocompatible constructs, membrane-based biosensors and devices, and analytical platforms for assaying membrane-based processes [14,43,68–70].

Here, we review a collection of recent experiments, which illustrate deliberate substrate–membrane coupling using simple structured surfaces exhibiting binary chemical and topographic patterns. These

experiments demonstrate the suitability of the supported membrane configuration in offering a direct interface-mediated control of lipid organization (e.g., mono- and bilayer morphologies), physical properties (e.g., curvature), and dynamical reorganization (e.g., membrane re-equilibration) and their indirect influences on many other membrane biophysical characteristics (e.g., phase separation, phase transition, membrane tension, etc.). Rather than producing an exhaustive list of relevant experiments, the current review aims to illustrate this idea through a collection of efforts from our and other laboratories.

2. Controlling membrane organization using chemically patterned substrates

Interfacial control of lipid organization is most obvious in free bilayers embedded in aqueous phases. Phenomenologically, the so-called hydrophobic effect serves as a primary driving force in orienting individual lipids into a bilayer motif [71]. It promotes polar head-group interactions with the interfacial water and screens the exposure of the hydrophobic acyl chains. This interfacial effect in conjunction with intermolecular van der Waals interactions, hydration forces, and electrostatic interactions determines the details of membrane organization within lipid bilayers. In this regard, use of substrates exhibiting markedly different interfacial properties should allow a control over membrane organization. Indeed, it is now well established that at high-energy hydrophilic surfaces, exposure to unilamellar vesicles results in the formation of single supported lipid bilayers [15,72,73]. In contrast, low-energy hydrophobic supports foster vesicle spreading by an entirely different mechanism, which consistently produces single lipid monolayers [74–78]. Based on the above, it seems likely that single substrates exhibiting predetermined variations in interfacial free energies should yield novel lipid organizations reflecting local variations in interface properties.

Static two-dimensional patterns of interfacial free energy are straightforward to prepare using chemical patterning methods. The surface chemistry of several bilayer-supporting substrates (e.g., clean silica, silicon, or mica) are easily manipulated using such techniques as optical lithography, micro-contact printing, dip-pen nano-lithography, or soft lithography to create patterns in the nanoscopic to microscopic range [47,79]. The basis for these methods is the covalent bonds that form between alkylsiloxanes and oxidized silica (e.g. SiO_2), which allow the self-assembly of uniform hydrophobic monolayers over the hydrophilic oxidized surface. Indeed, similar techniques have been used to deposit monolayers over coinage metals (Au, Ag, Cu) using alkanethiols as well [80]. However, standard vesicle fusion often fails to produce uniform films over such substrates, as the vesicles tend to adhere to the exposed metal without rupturing, producing inhomogeneous patches of intact vesicles [81,82]. Since the benefits to having a conducting surface as the membrane support would be numerous, a possible method for obviating this barrier to achieving uniform bilayers over coinage metals is discussed below.

A facile technique, which can be used to control the surface chemistry (i.e., wettability) of bilayer-supporting substrates, consists of deep UV-illumination of a self-assembled alkylsiloxane monolayer through a photo-mask, resulting in features of microscopic or macroscopic dimension [53,83]. Specifically, we allow the formation of an octadecyltrichlorosilane (OTS) monolayer on newly oxidized silica or silicon supports by classical solution-phase self-assembly [83,84]. The resultant monolayer is illuminated with an ozone-producing UV lamp (medium-pressure Hg discharge grid lamp in a quartz envelope) through a physical mask composed of chrome over quartz. As UV/ozone ablates the OTS monolayer in unprotected areas, this technique produces alternating regions of hydrophobic and hydrophilic character in any geometry available on a photo-mask [53].

When vesicle fusion or lipid spreading is conducted on surfaces of patterned OTS on SiO_2, lipid bilayers are produced in the hydrophilic regions, while lipid monolayers are produced in the hydrophobic areas where the OTS remains [16,53] (Fig. 1, panel 1). The result is a unique arrangement of mono- and bimolecular films, which makes possible the construction of complex biomembrane models. Interestingly, fluorescence recovery after photobleaching (FRAP) studies have shown that, while the individual mono- and bilayer regions exhibit lateral diffusion coefficients in the expected range, the two types of features are discontinuous. That is, diffusion is not possible *between* the mono- and bilayer films, as evidenced by FRAP data showing that fluorescence recovery does occur within, but not between features [53] (Fig. 1, panels 2 and 3). The blockade to inter-feature diffusion is thought to be due to the presence of a poorly characterized region, which supports neither mono- or bilayer lipidic films. This so-called "moat" region is lipophobic, but does support the adsorption of proteins, such as bovine serum albumin and streptavidin, and in this regard is similar to the hydrophobic OTS [53] (Fig. 1, panel 4). In this way, two-dimensional chemically produced patterns of differential interfacial energy or wettability can be translated into intricate mono- and bimolecular lipidic patterns exhibiting deliberate fluidity barriers. Notably, on patterned substrates with some 3D character (e.g., mesoporous silica), the barrier to lateral diffusion does not exist, and communication is possible between the mono- and bilayer regions at the top leaflet [85]. Thus, the level of lateral communication between the different regions of the patterned lipid film is tunable through manipulation of the substrate.

The unique character of the lipidic morphologies obtained here has many practical uses. The patterns of juxtaposed bilayers and monolayers derived from a single vesicular source on single substrates provide a general means to study many biophysical questions and design biomimetic platforms to array bilayer (or monolayer) specific functionalities. The variety of applications is demonstrated in the following two examples wherein the application of the construct provides new biophysical insights.

First, we find that co-existing lipid mono- and bilayer patterns provide a generic means to determine the leaflet-dependent compositional asymmetry within the supported membranes. In a recent study, we examined the leaflet-dependent partitioning preferences of glycolipid GM1 (monosialo ganglioside) and Texas Red-DHPE molecules in supported membranes. GM1, a native receptor for cholera toxin [86] and a raft-partitioning molecule [87], is widely used to characterize protein–sugar interactions and lipid rafts. Texas Red conjugated lipids are broadly employed as fluorescent probes to visualize cellular and model membranes. We presented compositionally symmetric POPC vesicles containing 2 mol% GM1 to patterned OTS templates and subsequently exposed the patterned lipid morphologies to a FITC (fluorescein-5-isothiocyanate)-labeled cholera toxin (B_5 subunit, CTB) solution. Representative ellipsometric and epifluorescence data shown in Fig. 2, panel 1 confirm a notably higher binding of cholera toxin to the bilayer regions of the sample. To analyze these observations, we recall that ellipsometric data suggest that the molecular packing densities in the outer leaflets of mono- and bilayers are comparable [23]. Furthermore, assuming that vesicles transferred to the hydrophilic and hydrophobic surfaces do not show preferential exclusion (or inclusion) of POPC or GM1, we infer a significant enrichment of GM1 in the distal leaflet (farther from the substrate) of the bilayer regions. This enrichment presumably occurs because of the substrate electrostatic-driven rearrangement of charged molecules during the vesicle spreading process. The underlying assumptions of (a) comparable GM1 densities accessible to cholera toxin binding in mono- and bilayer configurations; and (b) linear correlation between CTB binding and GM1 concentrations are described in considerable detail in the original source publication and details of these calculations are provided in the corresponding supplemental material [23].

Next, when the starting vesicles contain a small concentration of head-labeled Texas Red-DHPE (0.5–1 mol%) in addition to the GM1,

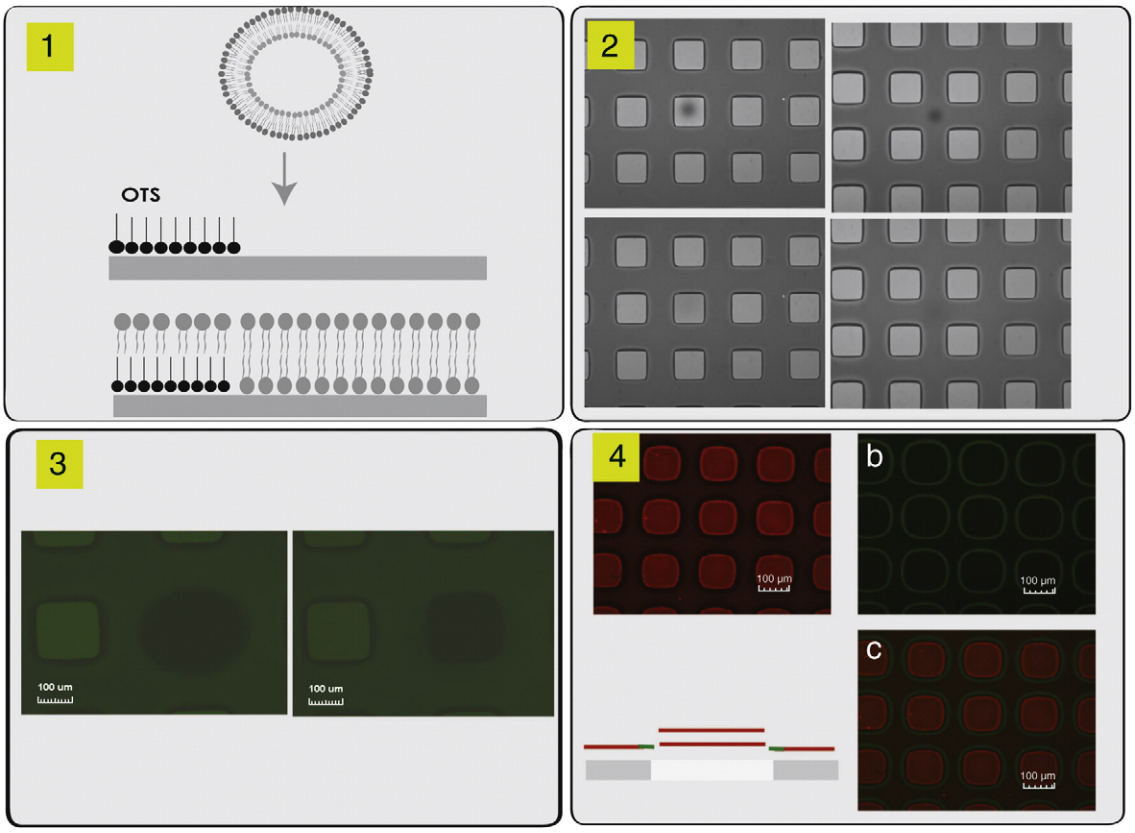

Fig. 1. Panel 1 shows a schematic representation of vesicle fusion over patterned OTS, producing a lipid bilayer in the exposed glass regions and a lipid monolayer over the OTS regions. Panel 2 shows a FRAP experiment resulting in fluorescence recovery in both the bilayer regions (bright squares) and the monolayer regions (grid pattern). Panel 3 shows a FRAP experiment indicating that fluorescence recovery does not occur between features. Panel 4 shows FITC-labeled BSA can adsorb to the "moat" region, similar to a bare OTS monolayer lacking lipids.
Adapted from Howland et al., JACS 2005 [53].

distinctly different fluorescence emission patterns emerge (Fig. 2, panel 2). Epifluorescence images in the FITC channel (1st image) reveal a markedly diminished intensity for bilayer regions despite greater amount of FITC-CTB. Because FITC (excitation, 490 nm; emission, 525 nm; 490/525) is known to be quenched efficiently when in close proximity to Texas Red (590/615) in lipid layers [88,89], the observed

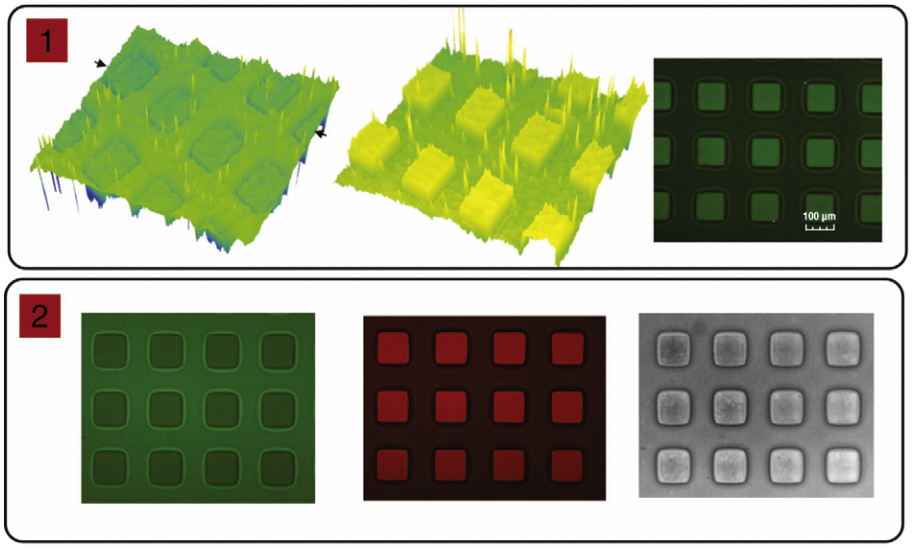

Fig. 2. Panel 1: The first image shows the ellipsometric thickness map of mono- and bilayer patterns consisting of 1 mol% GM1 and 99 mol% POPC in 75 mM PBS medium. The middle image shows the ellipsometric profile for the same sample after incubation with FITC-CTB. The third image represents an epifluorescence micrograph of an identical companion sample on glass revealing FITC (green channel) emission due to CTB adsorption. Panel 2: Epifluorescence images showing the green channel (FITC-CTB emission) and red (TR-DHPE emission) fluorescence patterns of a comparable sample containing 1 mol% TR-DHPE and 1 mol% GM1 lipid after incubation with FITC-CTB. The third image represents the FRET channel micrograph of a comparable sample.
Adapted from Shreve et al., Langmuir 2008 [23].

diminution in fluorescence intensity suggests Forster resonance energy transfer (FRET) between the two complementary probes. The FRET channel image in Fig. 2, panel 2 (3rd image) (490/615), corrected for bleed-through in our experimental geometry, confirms this inference. Because FRET efficiency decreases rapidly with the separation between donor and acceptor probes (Forster radius for FITC-Texas Red pair is ~5 nm), and a significantly higher amount of FITC-CTB is present in the bilayers, the observed enhancement of FRET signal can be explained in terms of a greater population of Texas Red-DHPE probe in the distal leaflet of supported membranes. Taken together, these results are consistent with the notion that many negatively charged lipids reorient such as to populate distal leaflets when organized as fluid bilayers on negatively charged substrates (e. g., silica). Furthermore, the applications above illustrate the general use of this construct to estimate partitioning preferences of specific molecules across the two lipid leaflets.

Second, the patterns of lipid mono- and bilayers can influence the attachment and growth processes of adherent cells. When human retinal pigment epithelial (ARPE-19) cells are presented with patterns of phospholipid mono- and bilayers, formed over patterned OTS substrates, they attach and grow in the monolayer regions in preference to the bilayer regions [84] (Fig. 3 panel 1). The resistance of bilayers to cell adhesion has been well documented [90,91], and has variously been explained by surface electrostatics (net electrical neutrality of the head-group), head-group hydration (strongly bound water), and lateral fluidity (lack of a firm anchor for the cell's focal adhesion) [90,92,93]. Since the mono- and bilayer system is created from a single stock of lipid (POPC), all of the above mentioned variables will be identical between the mono- and bilayer regions. Thus, such variables fall short in elucidating the differences between cell adhesion and growth over the two types of substrates. In addition, the lateral diffusion coefficients for lipids in both types of films are comparable [16,53] and fall in the range of published values for supported phospholipid bilayers (0.5–5 $\mu m^2/s$) [94]. One plausible explanation can be found in the undulatory motions available to the bilayers, which are significantly more restricted in the monolayer configuration [95,96]. The bilayers "float" over a thin (several angstroms) layer of water [19,20], whereas the monolayers are closely apposed with the underlying OTS monolayer. The OTS monolayer, in turn is covalently bound to the silica substrate, affording very little mobility normal to the plane of the lipidic film.

This difference in Z-axis mobility between mono- and bilayers was recently illustrated by the reaction of such lipidic films to the deposition of silica beads on the surface of each. Unlabeled silica spheres (~5 μm) were dropped over the surface of a mono- and bilayer grid pattern. We found that beads settle uniformly on mono- and bilayer regions at comparable densities. However, the lipid (POPC) bilayer, which was doped (1 mol%) with a fluorescently labeled lipid (TR-DHPE), was able to wrap partially the beads that fell in the bilayer regions, causing them to become fluorescent [97] (Fig. 3, panel 2). In contrast, the beads that settled onto monolayer regions remained non-fluorescent presumably due to the negligible out-of-plane mobility available to the monolayer [97]. Additionally, it was confirmed that the effect was related to membrane mobility by utilizing DMPC ($T_m = 24$ °C) for the mono- and bilayer system. When the lipid was in the gel phase (15 °C), none of the settled silica spheres became fluorescent; indicating that wrapping of the spheres was inhibited in both mono- and bilayer regions. In contrast, when the lipid was in the liquid crystalline phase (30 °C), a pattern similar to that obtained with POPC (at room temperature) was seen: only those spheres that settled onto the bilayer regions gained fluorescence and those on the monolayer remained non-fluorescent. Together, with estimates of

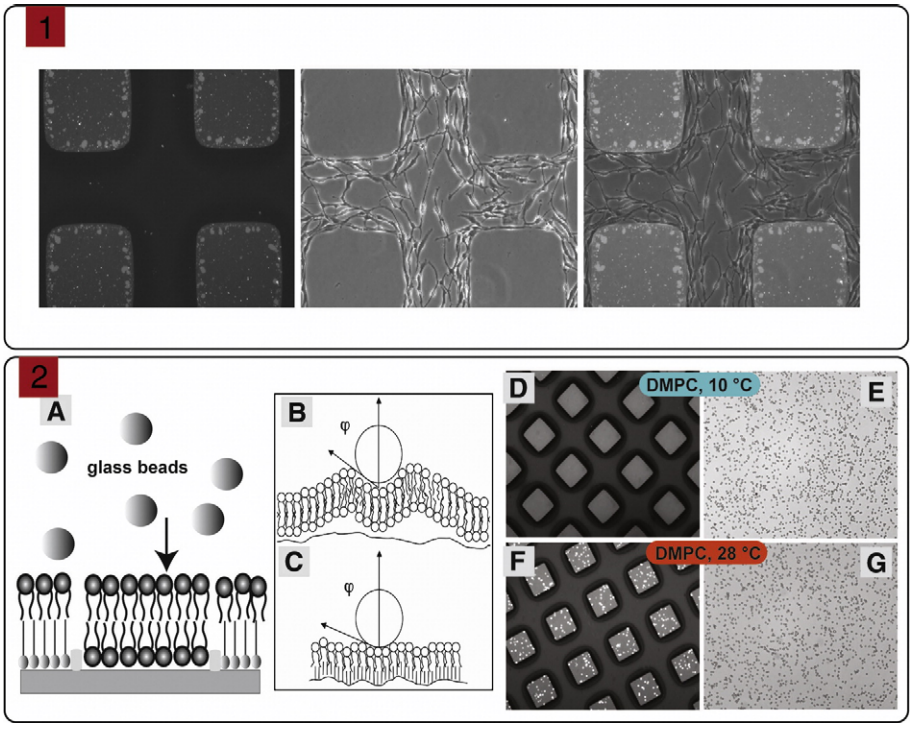

Fig. 3. Panel 1 shows ARPE-19 cells growing on a pattern of phospholipid (POPC + 1% Texas Red-DHPE) bilayers (bright 250 × 250 μm squares) surrounded by a monolayer grid (dim regions) as imaged by fluorescence microscopy (1st image); phase contrast microscopy (2nd image); or both together (3rd image). Panel 2: Discrimination of monolayer and bilayer morphologies using beads. A) Schematic representation of the monolayer/bilayer lipid assembly on a patterned OTS substrate with glass beads settling on the surface of the lipid films. B, C) Schematic illustrating partial lipid wrapping of a glass bead, which is available in the bilayer configuration, but not in the monolayer configuration. D, E) Fluorescence and bright-field images immediately after 5.66 μm bead sedimentation on DMPC at 10 °C. F, G) Fluorescent and bright-field images 15 min after heating the sample to 28 °C show fluorescent beads in the bilayer regions.
Adapted from Oliver et al., Langmuir 2009 [84]; Dixit et al., ChemPhysChem 2006 [97].

fractional wrapping and area expansion of the bilayer derived from fluorescence intensity analysis detailed in Dixit et al. [97], we inferred that bilayer wrapping of beads, above the transition temperature, reflects the out-of-plane mobility available to bilayer lipids in the liquid crystalline phase [97].

Besides the hypothesis that such undulatory motion is responsible for the resistance of lipid bilayers to cell adhesion, alternative possibilities also exist. For instance, the differences noted above between cell adhesion seen on mono- and bilayers might be due to differences in the defects in the mono- and bilayer lipid films through which adhesive proteins could adsorb to the underlying substrate. Thus, additional work will be necessary to fully decipher the mechanism(s) responsible for the distinct pattern of attachment onto the mono- and bilayer substrates. Nevertheless, the ability to confine the adhesion and growth of living cells through mono- and bilayer patterns on lipidic substrates may offer additional biological applications. For instance, the lipid layers could deliver membrane-based stimuli (e.g., ceramide) to adhering cells, and thus elicit cellular responses in a controlled manner. Similarly, control of other physical properties (e.g., curvature, tension, and fluidity) of the underlying lipid layers could provide additional means to modulate cell-surface interactions or behavior. Such exploitation of the membrane/plasma membrane interface in this way allows a unique approach for the study of cell-surface signaling.

In summary, the rupture and spreading of vesicles onto chemically structured surfaces result in a unique lipid structure revealing template-induced assembly of co-existing lipidic phases. In these constructs, the spontaneous separation of the fluid bilayer regions from the fluid monolayer regions, by the moat interface, provides a simple method to compartmentalize membrane mobility. Furthermore, the co-existing bilayer/monolayer morphologies derived from single vesicular sources are particularly attractive for studies spanning biophysical processes and cell-surface signaling. Furthermore, such lipid structures also offer a new self-assembly strategy for synthesizing large-scale arrays of functional sub-structures including ion channels, membrane proteins [69], and cells.

3. Controlling membrane curvatures using structurally corrugated substrate interfaces

Since the natural world cannot, in general, be constrained to two dimensions, it is imperative that regulation of the substrate membrane interface extends to the physical realm as well. Thus, appropriate models for characterizing natural phenomena, as well as exploiting subsets of biomolecules toward specific applications, must include the capability to exist in three dimensions. A significant example of the three-dimensional nature of membranes is represented by the many instances of curvature found in living organisms. Several essential biological functions including vesicular budding, viral interactions, mitosis, and membrane fusion proceed via structural intermediates that display well-defined transient curvatures [98–101]. Inside the cell, quasi-static curvatures also exist in microvilli and as folded membranes in many organelles (e.g., cilia, thylakoids) [102–104]. Living cells stabilize (or modulate) their local membrane curvatures by concentrating or dynamically recruiting molecules with intrinsic curvatures, (e.g. lipids, membrane proteins, amphiphilic helical peptides, etc.) or via time-dependent external scaffolding mechanisms including cytoskeletal (re)polymerization or motor protein activity [100].

The investigation of lipid bilayers deposited over structures consisting of regular repeating curvatures can be greatly aided by two approaches developed in our laboratory. Both are static three-dimensional structurally corrugated substrates. *The first* approach involves releasing stretched elastomers after surface oxidation, which results in multiple nested orders of periodic surface curvatures over macroscopic areas of elastomeric surfaces [105]. Briefly, uniaxially stretched flat poly(dimethyl) siloxane (PDMS) surface is exposed to an ozone-generating, short-wavelength ultraviolet (UV, 184–257 nm) radiation for 30–60 min. Subsequent removal of the strain results in periodic wrinkling in a direction perpendicular to the strain. At least three nested orders of curvature are evident by atomic force microscopy [105] in good general agreement with previous studies [106]. Incubating these patterned curved surfaces with small unilamellar vesicles of fluid phospholipids (e.g., POPC) results in the formation of contiguous phospholipid bilayers, which roughly follow the substrate topography for microscale and higher periodicities [105]. Epifluorescence imaging and FRAP establish homogeneity and lateral fluidity of the supported bilayer films [105] (Fig. 4). These surfaces provide useful opportunities for templating curvatures (as well as nested hierarchy of curvatures) onto fluid lipid membranes.

The second, a face-centered cubic (f.c.c.) lattice composed of silica colloids in the nano- or microscopic range, can be constructed through physical confinement [107,108]. Briefly, the colloidal crystal is produced by sandwiching a small volume (10–20 μL) of a colloidal solution near the critical concentration (~45% v/v) between one hydrophilic and one hydrophobic plate, and allowing for slow evaporation of solvent over a period of 3 days [107]. Conducting vesicle fusion on colloidal crystals formed in this way produces variable results depending upon the size of the colloids. If small colloids (e.g., 330 nm) are utilized, 100-nm vesicles will fuse and spread, creating a laterally contiguous lipid bilayer with a regular pattern of curvature [108] (Fig. 5, panel 1). In contrast, if large colloids (e.g., 5.6 μm) are used, FRAP studies of the fluorescent lipid adhering to the surface reveal that long-range fluidity is absent [108]. This discrepancy can be explained by the differences in the dimension of the interstitial spaces. The interstices for the 330-nm colloids are estimated at $\leq$49.5 nm [108], which would disallow the 100-nm lipid vesicles from penetrating the surface. The vesicles, thus, presumably rupture at the surface of the colloidal crystal, forming the continuous bilayer. Conversely, the vesicles are likely to pass through the interstitial spaces of the 5.6 μm colloidal crystal, allowing "wetting" of the internal colloids, rather than formation of a continuous bilayer at the surface [108]. Thus, the use of the small colloids in the preparation of the colloidal crystal allows the investigator to design, within certain physical constraints, a contiguous bilayer that follows the topology of a curved substrate of specific dimensions.

The physical corrugation described here can be combined with the chemical patterning described above to generate an additional level of investigative control. For example, the nanoscale colloidal crystals can be exposed to the silanization and deep UV exposure treatment. Interestingly, coating the colloidal crystal with OTS, followed by UV-patterning under a photo-mask, and subsequently exposing it to vesicle fusion, results in a three-dimensional system whereby the hydrophilic regions support a lipid bilayer and can fill with water, whereas the hydrophobic regions support a monolayer, but remain dry inside [108] (Fig. 5, panel 2). Preliminary results from incorporation of the ion-conducting peptide gramicidin into bilayers supported by colloidal crystals show this technique has great potential for the incorporation of ion channels into the bilayer regions, and the detailed study of their function by monitoring the characteristic photonic band gaps of the nanoscale colloidal crystals.

4. Controlling membrane dynamics using switchable substrate interfaces

The two general types of substrates described above, chemically patterned and structurally corrugated, both fall into the category of static supports, in that the substrate remains physically unchanged following bilayer deposition. An alternative strategy is the use of substrates with dynamic properties. Engineering the interface between the substrate and the lipid bilayer takes on additional complexity in such cases, as the physical properties of the component parts of the interface

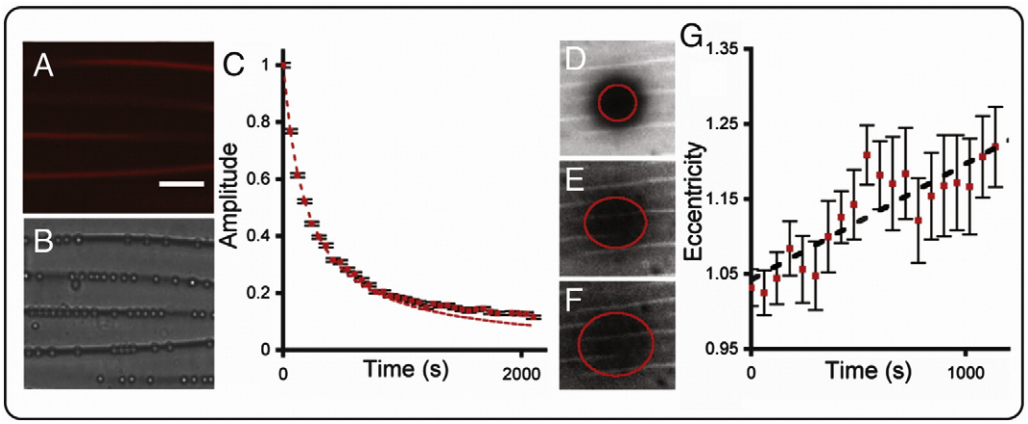

Fig. 4. Phospholipid bilayers on wrinkled elastomers. (A,B) Wide-area epifluorescence (A) and bright-field optical transmission (B) images of a POPC bilayer (doped with 1 mol% Texas Red-DHPE) on a pre-wrinkled PDMS elastomer. The image in (B) is acquired after 5 μm beads are allowed to settle onto the bilayer. The white bar scales to 50 μm. (C) Normalized Gaussian amplitude of a bleached FRAP spot's intensity depression as a function of time, showing fluid recovery. Dashed line is a fit to the expected recovery profile. (D–F) Selected frames from a sequence of fluorescence images showing recovery dynamics of a circular spot photobleached into a POPC bilayer (doped with 1 mol% TR-DHPE) deposited on the wrinkled ox-PDMS substrate at representative time intervals $t = 0$, 600, and 1020 s after photobleaching). The red trace is an ellipse fit to the spot edges. Images scale to 112 × 112 μm. (G) Temporal evolution of eccentricity of the photobleached spot in panels (D–F).
Adapted from Sanii et al., Nanoletters 2008 [105].

(e.g., viscosity and osmolarity of the aqueous cushioning layer, phase behavior of the membrane leaflet proximal to the substrate, etc.) change over time, and thus the dynamics of the system must also be considered. Below, we describe two recent examples, one involving dynamic introduction of curvature at the membrane–substrate interface and the second involving time-dependent dissolution of carbohydrate substrates.

First, curvatures in living systems are highly dynamic. During essential cellular processes such as growth, division, and motility, cellular membranes undergo dramatic conformational changes. Such dynamic membrane remodeling proceeds via incorporation of curvature-sensitive lipids and via interactions with curvature-generating and curvature-sensing proteins [100]. To test if the flexible PDMS-based support described above could be used as a topographically dynamic substrate to induce remodeling in the lipid bilayer that it supports, we carried out a simple proof-of-concept experiment [105]. Briefly, a stretched (30%) flat PDMS elastomer is subjected to a typical UV treatment (see above) to generate a stiff oxidized-PDMS surface. In two parallel experiments, unilamellar vesicles composed of dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, $T_m = 41$ °C) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, $T_m = -2$ °C) are fused to the substrates at temperatures above their respective T_m values and then allowed to equilibrate at room temperature (24 °C), all while the PDMS is still stretched. Epifluorescence images reveal a homogeneous bilayer for each sample, consistent with the formation of a planar supported bilayer on flattened, stretched oxidized-PDMS. A large circular spot (650 μm diameter) spanning multiple topographic corrugations is then photobleached into both DPPC and POPC specimens. The stretch is released and, as expected, the substrate deforms in two parallel modes: (1) the bulk PDMS tries to laterally restore its original shape by compressing in the stretched direction and elongating in the perpendicular one determined by the PDMS's Poisson ratio of 0.5, and (2) the

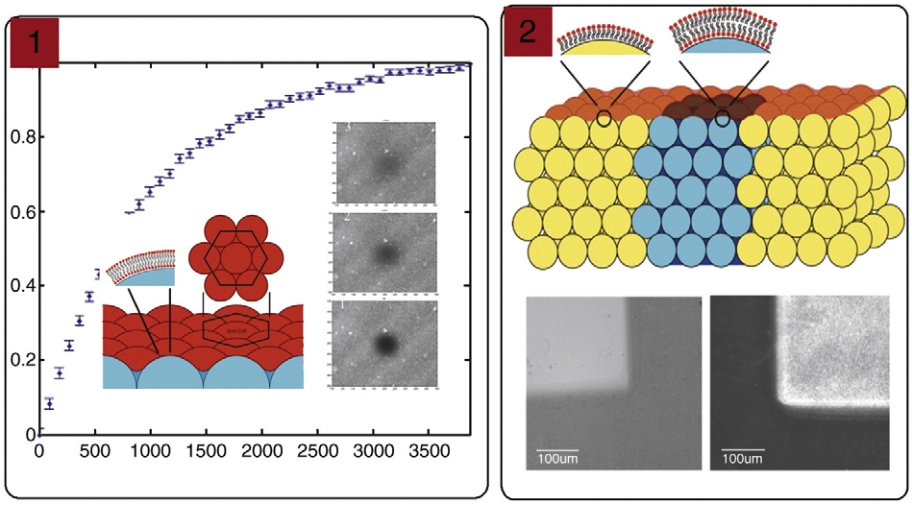

Fig. 5. Panel 1: A FRAP fractional recovery plot of a Texas Red doped POPC bilayer on a 330 nm colloidal crystal. Insets: 200 × 160 μm epifluorescence images of a bleached spot (40 μm diameter) at times, $t = 0$, 630, and 1530 s and a cartoon illustrating a bilayer on top of a nanocolloidal crystal. Panel 2, top: A cartoon illustrating spatially patterned columns of hydrated, hydrophilic colloids (blue) in hydrophobic surroundings (yellow) of a colloidal crystal. Bottom left: A 10× bright-field image of UV-patterned OTS-coated 330 nm silica crystal in water. The darker region corresponds to an unhydrated, OTS-coated region (band gap < 605 nm), and the brighter region is the hydrated region (band gap < 658 nm). Bottom right: A 10× fluorescence image of a TR-DHPE doped POPC film on a patterned colloidal crystal.
Adapted from Brozell et al., JACS 2006 [108].

mismatch in the equilibrium strains of the stiffened surface skin and the bulk PDMS produces periodic out-of-plane wrinkles, confirmed by bright-field microscopy. Simultaneously, a dramatic transformation in the shape of the fluorescence spot is observed for both POPC and DPPC bilayers: in both cases, the initial circular spot transforms into an elliptical one, with the minor axis in the direction of the released stretch (Fig. 6) [105]. Both bleached spots recover when heated to above their respective melting temperatures, indicating that the membranes remain laterally contiguous throughout the wrinkling transition. FRAP analysis of POPC membranes that have undergone a wrinkling transition indicates an azimuthally averaged diffusion coefficient of $1.7 \pm 0.4\ \mu m^2/s$. The recovery was anisotropic, consistent with membranes deposited on pre-wrinkled substrates. Together, these results establish that substrate deformation and wrinkling remodel membrane bilayers in the fluid (POPC at 24 °C) and, more remarkably, in the gel (DPPC at 24 °C) phase. These simple preliminary results suggest that dynamic variations of substrate topography trigger spatially patterned mesoscale restructuring of the bilayer accompanied by curvature-dependent spatial reorganization of membrane molecules.

This ability to dynamically impose curvatures on supported bilayers and observe the attendant re-equilibration may be useful for fundamental studies of many curvature-induced dynamic reorganizations and their functional consequences. Because patterns of curvatures can stabilize heterogeneous distribution of molecules within fluid membranes, these model systems may also provide a generic means to create sustained molecular gradients and carry out spatial separations of membrane-compatible amphiphiles [109]. Our approach however has many important limitations. PDMS generates one-dimensional wrinkles. In contrast, curvatures in cellular membranes are often 2D with non-zero Gaussian curvatures. Second, the proximity of the substrate and practical difficulties in achieving a precise range of curvatures in PDMS also introduce additional complications. Nevertheless, qualitative assays of preference of certain lipid-types and domains for curvature should be possible using our construct.

Second, the ability to develop substrates, which exist only as temporary supports during the creation of the lipid bilayer, seems particularly valuable. Such ability could afford studies of dynamic membrane reorganization, as the interfaces surrounding the bilayer change their properties. An extraordinarily powerful biosynthetic tool, in this regard, is the use of carbohydrate glasses to control interfacial activities of membrane components. Glass-forming disaccharides, such as trehalose, are found in high concentrations in many anhydrobiotic organisms and serve a critical role in preserving the membrane bilayer structure under the severe stresses associated with dehydration [110,111]. Interestingly, trehalose glasses, which are uniformly hydrophilic, have proved to be excellent substrates for bilayer formation by vesicle fusion [112]. However, the glasses devitrify upon contact with water, entirely altering their physical structure, opening the possibility that such compounds may be exploited as temporary interfacial membrane supports.

The dynamic nature of such glassy supports would be useful under circumstances where bilayer formation required provisional scaffolding. As mentioned above, the formation of a fluid, contiguous lipid bilayer over the surface of coinage metals would open the door to

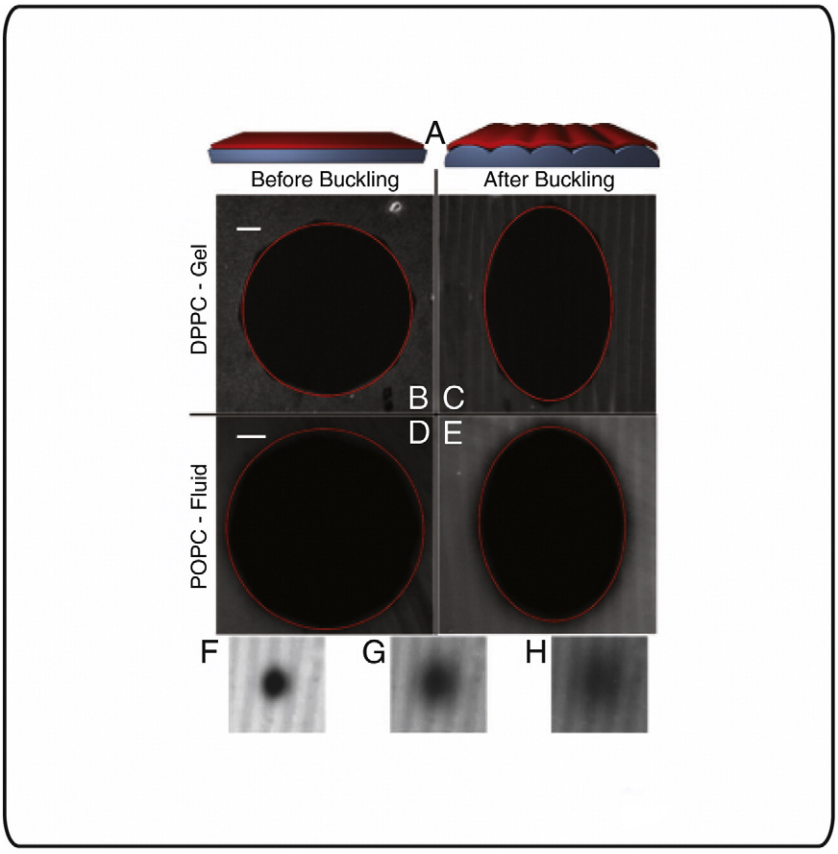

Fig. 6. Active membrane remodeling via surface wrinkling. (A) A cartoon depiction of a lipid bilayer (red) supported on stretched planar ox-PDMS before (left) and after (right) the stretch is released. (B–E) Representative epifluorescence images showing macroscopic membrane remodeling after release of the mechanical stretch. Left column: Circular spots bleached into membranes deposited onto stretched ox-PDMS before release. Right column: Spots after the stretch was released and the substrate has wrinkled. Top row: DPPC lipids in a gel state. Bottom row: POPC lipids in a fluid state. The white bar scales to 100 μm and is consistent across rows, and red traces are least-square-fits of ellipses to the bleached areas. (F–H) The recovery of a spot photobleached into a lipid bilayer that underwent remodeling via substrate wrinkling. The images are 30 s, 400 s and 1100 s after bleaching respectively, and scale to 65 μm a side.
Adapted from Sanii et al., Nanoletters 2008 [105].

numerous conductive studies and devices. In this regard, initial studies suggest trehalose glass can be used as a "sacrificial" substrate, to allow the deposition of a phospholipid bilayer over a surface that would otherwise only adsorb the intact vesicles [81,112]. Proof of principle for this technique has been demonstrated with gold substrates. Fluorescently doped vesicles adhere inhomogeneously to plasma-cleaned gold, whereas if a trehalose glass is first deposited on the gold substrate (by dehydration of a trehalose solution at 100 °C), vesicle fusion results in the formation of a laterally contiguous lipid bilayer as evidenced by FRAP studies [112] (Fig. 7, panel 1).

Further study will be necessary to determine the useful life-times of membranes constructed in this manner, each of which will depend on the composition of the underlying substrate, the concentration, viscosity, and diffusion of carbohydrate between the substrate and the bilayer, and whether the sugar is an absolute requirement for maintenance of the bilayer. Nevertheless, possible future applications of the sacrificial carbohydrate layer are numerous.

Two such areas are currently under investigation in our laboratory. In the first, trehalose glasses are being used in the construction of colloidal crystals. Additionally, sacrificial trehalose glasses are being used in order to suspend lipid bilayers over wells created by photoresist in elastomeric substrates. Such approaches are likely to enable much progress in the ongoing effort to incorporate ion channels, G-protein coupled receptors, or other key membrane proteins into phospholipid bilayers, while retaining function and lateral diffusivity. Further, the ability to incorporate functional membrane proteins into bilayers suspended over individually addressable aqueous compartments opens new avenues for high-throughput analytical systems [69,113].

In addition, the importance of carbohydrate glasses to the field of SLB will be immediately obvious to anyone who has considered the potential liabilities associated with storing and/or shipping biotechnologic devices composed primarily of a supported membrane, whether containing or devoid of proteins. The lifetime of a SLB is on the order of a few hours, and these fragile structures delaminate from the surface with the slightest exposure to an air bubble [114]. Some attempts have been made to protect SLBs with protein or poly (ethylene glycol) coatings [114,115], or polymerization [116–118], such that removal from the aqueous environment does not destroy the membrane. However, the presence of these layers themselves may affect structural organization of membrane components and also influence membrane biophysical properties (e.g., lateral fluidity).

Two recent reports have shown that vitrification of trehalose can protect SLBs from moderate [115] to severe dehydration [112]. In fact, when trehalose is included at the interfaces of both membrane leaflets, the severe stresses of extreme drying and rehydration cause no significant change to the long-range lateral lipid mobility in the hydrated state, as confirmed by FRAP measurements [112] (Fig. 7, panel 2). Thus, there are important implications for the use of carbohydrate glasses in helping to transform the many possibilities of membrane/protein-based bio-devices from mere potential into a practical reality.

In summary, both types of investigations, the study of bilayers deposited on stretched elastomers and the study of bilayers formed over carbohydrate glasses, open new avenues of research into substrates with dynamic properties. Such investigations include an added level of complexity, as the dynamics of each physical characteristic will affect the interfacial regions of lipid membranes, and thus the overall properties of the bilayer.

5. Outlook

The variety of useful membrane configurations obtained by substrate-induced templating of vesicle fusion and lipid spreading illustrated here represent a small sub-set of myriad interesting and useful possibilities to control membrane structure, assembly, and dynamics. They extend the well-established notion of template-directed material design in solid state materials (e.g., crystallization of inorganic materials [119], self-assembly of colloids [120], and phase separation of polymer mixtures [121]) to complex fluids, of which the

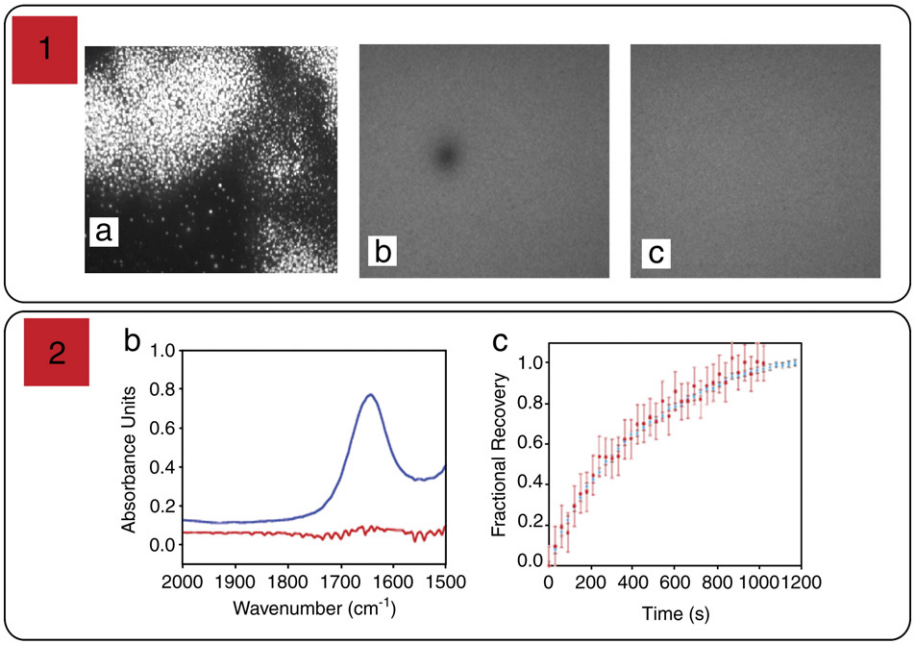

Fig. 7. Panel 1: (1st image) Fluorescence micrograph of POPC (+1 mol% Texas Red-DHPE) vesicles adhering inhomogeneously to plasma-cleaned gold surface. (2nd image) Fluorescence micrograph of POPC (+1 mol% Texas Red-DHPE) bilayer, deposited by vesicle fusion to a pre-formed trehalose glass on top of plasma-cleaned gold surface, frame 1 after photobleaching. (3rd image) Same as (2nd), frame 23 (=12 min after photobleaching). Panel 2: (1st image) Attenuated total reflection infrared spectra showing the O–H bending vibration at ~1643 cm^{-1} for trehalose samples before (upper trace, blue) and after (lower trace, red) incubation at 100 °C for 24 h. (2nd image) Fractional fluorescence recovery after photobleaching for a POPC bilayer deposited on trehalose glass: trace 1 (●, blue), initial; trace 2 (■, red), after drying and rehdyration.
Adapted from Oliver et al., Lab On a Chip 2008 [112].

lipid bilayer is a powerful example. The efforts reported to date set the stage for many future directions. Some examples are outlined below.

First, the efforts reported to date have focused on the use of various substrates and substrate property patterns to design novel membrane configurations. These initial studies suggest that the systematic tailoring of properties of support surfaces including substrate charge, wettability, and topography can be used to map out elemental "phase diagrams" for how interfacial properties determine membrane biophysical properties. *Second*, we envisage future studies employing three-dimensionally structured templates that "mold" lipid organization into variety of three-dimensional lipid mesophases (e.g., cubic, hexagonal, and multilamellar). In living systems, such structures often emerge transiently, for instance, during membrane fusion [99,122] and cell division [123]. Systematic studies of such temporal membrane sub-structures, which give rise to complex molecular sorting and localization of selected membrane functions, are sparse. We anticipate that the use of stable, three-dimensionally scaffolded SLB constructs should provide a useful platform to systematically dissect structure–function relations in these transient membrane mesophases. *Third*, the use of dynamically tunable and sacrificial substrates to template membrane dynamics, alluded to above, is still in its infancy. Such substrates offer a tremendous advantage over static structures in imposing new dynamic interfacial constraints, and thus offer a window into how membranes utilize their structural supramolecular landscapes to organize and reorganize their functions. *Finally*, it seems intriguing to devise membrane self-assembly processes that draw upon notions now well established in biomineralization. Specifically, biomineralization utilizes cooperative and/or hierarchical organization of both the templating biomolecules (e.g., proteins and lipids) and the inorganic crystallizing species (e.g., abalone shell calcite and diatom silica) to design intricate and biologically important hybrid (organic/inorganic) structures [124–126]. The majority of SLB configurations used to date employ preformed substrates. The possibility of concomitant formation of the substrate and the lipid bilayer may open interesting possibilities to devise novel biomimetic structures — especially those with relevance to transient sub-structures that emerge during the life of the cellular membrane.

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