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Solid supported lipid bilayers: From biophysical studies to sensor design

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Abstract

The lipid bilayer is one of the most eloquent and important self-assembled structures in nature. It not only provides a protective container for cells and sub-cellular compartments, but also hosts much of the machinery for cellular communication and transport across the cell membrane. Solid supported lipid bilayers provide an excellent model system for studying the surface chemistry of the cell. Moreover, they are accessible to a wide variety of surface-specific analytical techniques. This makes it possible to investigate processes such as cell signaling, ligand–receptor interactions, enzymatic reactions occurring at the cell surface, as well as pathogen attack. In this review, the following membrane systems are discussed: black lipid membranes, solid supported lipid bilayers, hybrid lipid bilayers, and polymer cushioned lipid bilayers. Examples of how supported lipid membrane technology is interfaced with array based systems by photolithographic patterning, spatial addressing, microcontact printing, and microfluidic patterning are explored. Also, the use of supported lipid bilayers in microfluidic devices for the development of lab-on-a-chip based platforms is examined. Finally, the utility of lipid bilayers in nanotechnology and future directions in this area are discussed.

Contents

1. Introduction

Phospholipid bilayers closely resemble cell membranes in some key respects. For example, they retain two-dimensional fluidity and can be an excellent environment for presenting membrane proteins. Model bilayer systems allow for the investigation of biological processes that occur at the cellular level, providing information about ligand–receptor interactions [1–4], viral attack [5,6], and cellular signaling events [7–9]. In the 1960s Mueller et al. developed the first system for the investigation of the electrical properties of a planar phospholipid bilayer [10,11]. This system, usually referred to as a black lipid membrane, consisted of phospholipid molecules painted across a 1 mm hole between two solution chambers. Twenty years later Tamm and McConnell deposited lipid membranes directly onto solid supports [12]. In 1997 Boxer et al. pioneered the partitioning of supported phospholipid bilayers into lithographically patterned corrals [13]. This led to the development of individually

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addressed arrays of solid supported phospholipid bilayers by Cremer and Yang [14] and sensor arrays for the study of cell adhesion by Groves et al. [15].

Phospholipid membranes have been combined with microfluidic systems for the development of powerful sensor applications. This includes on-chip immunoassays for investigating the kinetics and thermodynamics of antibody binding to antigens presented on bilayers contained within the microchannels [3,4], as well as bilayer coated microchannels which were used to present immobilized enzymes for the rapid determination of enzyme kinetics [16]. The utility of laminar flow within microchannels has also been used to facilitate the patterning of lipid bilayer arrays within microfluidic systems [17,18].

In certain instances black lipid membranes and even lipid vesicles in bulk solution have an advantage over solid supported phospholipid bilayers. For example, they avoid direct contact with an underlying substrate that can potentially be problematic for the presentation of transmembrane proteins. They also allow solution phase access to both sides of the membrane. However, they are less stable than supported membranes, harder to manipulate chemically, and are far less accessible to surface specific detection techniques. Therefore, it would be desirable to develop methods to appropriately embed transmembrane proteins into supported bilayers. To this end, Spinke et al. laid the foundation for polymer supported phospholipid bilayers on planar solid substrates [19]. It was found that thin polymer films could couple bilayers with a large variety of materials such as metal films, oxides, and semiconductor electrodes. Adding a polymer layer between an underlying substrate and the phospholipid bilayer can be achieved by the use of either a cushioning polymer film [20–25] or the direct tethering of the membrane to a lipid presenting polymer or peptide layer [26–31]. Other effective surface modification strategies include self-assembled monolayers (SAMs) [32,33] and the use of adsorbed or bound proteins as a cushioning layer [34–39]. To this day, however, there is not yet a completely satisfactory supported membrane system for the presentation of transmembrane proteins with both large extracellular and intracellular peripheral domains.

Due to their amphiphilic nature, phospholipid membranes are capable of organizing not only themselves but also associated proteins, nanoparticles, as well as other species either within the membrane or at its surface. Furthermore, phospholipid vesicles can be formed into nanoliter sized compartments and tubules providing molecular confinement and transport [40]. Such compartments can also be used as nanoreactors for enzymes [41–44]. Phospholipid structures can even serve as templates for nanostructure fabrication by imparting controlled positioning and growth of metal layers, proteins, and polymers into the system of interest [45,46]. Perhaps the most elegant use of phospholipid membranes in nanodevice applications involves their employment in sensor platforms. Pore- forming proteins and peptides can be chemically modified or genetically engineered, giving the user unprecedented control over membrane binding and transport properties [47]. This has allowed stochastic sensing of analytes down to the single molecule level [48–50].

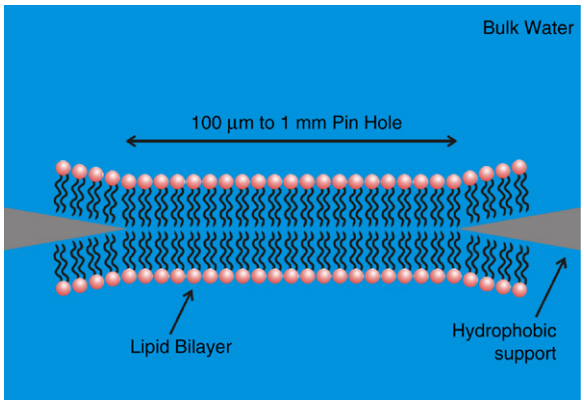

Fig. 1. Illustration of a black lipid membrane. The phospholipid membrane spans a 100 µm–1 mm pin hole in a hydrophobic support.

There are a variety of phospholipid membrane systems, supports, and detection schemes that can accommodate a host of applications. When making a choice of membrane platform, it is necessary to consider the analyte of interest. For example, solid supported bilayers on glass substrates are often sufficient for presenting small ligands for the study of multivalent interactions with extracellular proteins. On the other hand, if one wishes to incorporate transmembrane proteins or pore-forming toxins into the bilayer, it may be necessary to use a polymer cushioned bilayer or a black lipid membrane to prevent protein denaturation on the underlying substrate. Almost equally important is the detection scheme. A conducting substrate is required if the sensor design calls for a direct electrical measurement. In this case the bilayer can be supported on an indium-tin-oxide (ITO) electrode or even a gold electrode if an appropriately hydrophilic alkanethiol monolayer is employed. A good review of phospholipid membranes on solid surfaces is Ref. [51].

2. Black lipid membranes

The black lipid membrane derives its name from its appearance by optical microscopy. When Mueller et al. observed the formation of the first black lipid membranes [10,11] from extracted brain lipids, they noted interference bands giving rise to color in the membrane. This interference effect disappeared during the thinning of the painted lipid mass and is thought to indicate the formation of a single bilayer membrane, as shown in Fig. 1. An excellent resource on black lipid membranes is Ref. [52].

Several methods of producing black lipid membranes exist. All involve the formation of a membrane over a small aperture usually less then 1 mm in diameter. The hole is formed in a hydrophobic material such as polyethylene or Teflon and is usually part of a wall separating two compartments that can be filled with aqueous solution, each containing a reference electrode. Two of the most popular methods for BLM formation involve the painting of the lipid solution over the aperture [10] and the formation of a folded bilayer [53]. The result of either method is a bilayer suspended over the aperture with an aqueous compartment on each side.

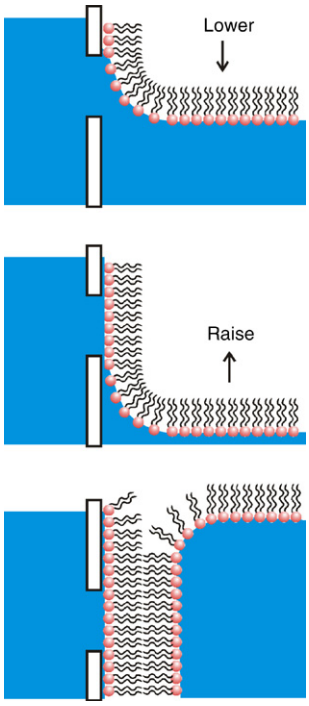

Fig. 2. The formation of a folded lipid bilayer. The solution on the side containing a lipid monolayer is slowly lowered and then raised. This deposits a monolayer with each pass producing the black lipid film.

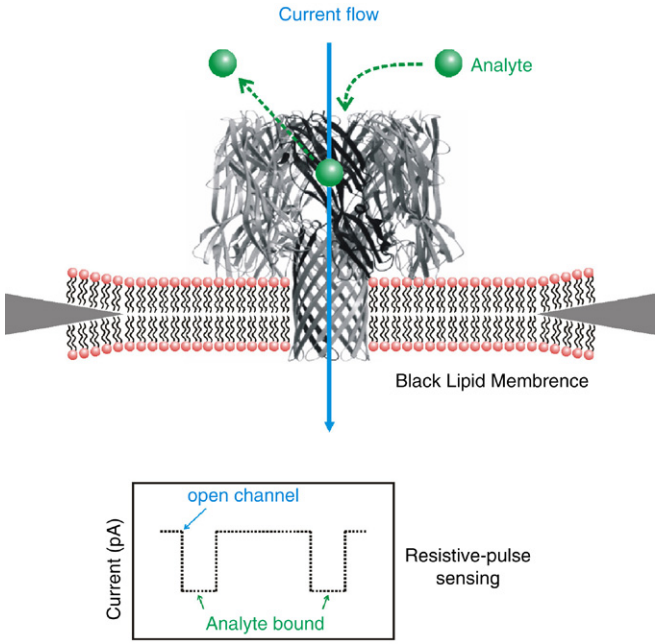

Fig. 3. Stochastic sensing with black lipid membranes. A pore protein such as α-Hemolysin can be used to sense single molecule binding within the protein's ion channel. The binding process is observed by a decrease in the current flowing through the pore in the presence of the analyte.

The painting of a black lipid membrane is carried out with a small artist paint brush. Typically a 1%–2% phospholipid solution in an organic solvent, such as n-decane or squalene, is painted across the hole under an aqueous solution. The deposited lipid mass thins as it spreads, forming the black lipid membrane. This methodology has remained basically unchanged over the decades [10]. The formation of folded lipid bilayers requires a cell with two compartments separated by a small aperture and the solution levels in each compartment must be controlled independently (Fig. 2). Both compartments are filled with the desired aqueous solution and a monolayer of phospholipid material is spread on top of one of the compartments. The solution level in the compartment containing the lipid monolayer is slowly lowered below the aperture and raised again. This deposits a monolayer on each pass to form the completed bilayer membrane [53].

Since their advent, black lipid membranes have been used to investigate various biophysical processes. One of the most important is the formation of ion channels in phospholipid bilayers by peptides [54], proteins [55,56], antibiotics [57], and other pore-forming biomolecules. Of particular interest for creating nanodevices is the insertion of single protein pores for use as stochastic sensors (Fig. 3). This has been accomplished by Gu et al. through the use of genetically modified α-hemolysin [49]. Naturally occurring α-hemolysin, which is composed of seven identical subunits, is an exotoxin produced by *Staphylococcus aureus* bacteria [58]. Through the use of genetic modification, α-hemolysin mutants were created which can non-covalently capture a cyclodextrin molecule within its pore. A current change at fixed voltage is measured when the cyclodextrin inserts into the channel due to a restriction of the pore cross-section. The current is further attenuated by the binding of a guest molecule in the cyclodextrin binding pocket. The binding and unbinding of small organic molecules within the cyclodextrin/α-hemolysin pore can ultimately be measured at the single molecule level via this process [49].

This same methodology has been applied to the stochastic sensing of divalent metal cations [47] and cell signaling molecules [48]. Polyhistidine motifs are known to strongly interact with divalent cations and are often employed in the purification of recombinant proteins. Pores designed to stochastically detect divalent metal cations were genetically engineered to present a short peptide sequence of four histidines inside the α-hemolysin pore [50]. A similar approach was used in the detection of cell signaling molecules. Pores were engineered with a ring of 14 arginine residues on their inside surface. It was shown that the phosphate groups on inositol 1,4,5-trisphosphate, a second messenger, interact with the ring of arginines, effectively blocking the pore [48].

Current measurements across a modified α-hemolysin pore show that the frequency of binding events relates to the concentration of the analyte. The amplitude of the current modulation together with the duration of time an analyte spends in the channel allows for specific identification of a given species [59]. Since only one molecule can fit into the channel at a time, analyte identification can be preformed for individual blocking events. This means that the same pore can be used in a sequential fashion to detect a variety of analytes.

As noted above, black lipid membranes are suspended in solution and there are no unwanted interferences of the membrane with an underlying support. The absence of such a support also means that transmembrane proteins suspended within the phospholipid bilayer remain fully mobile and active.

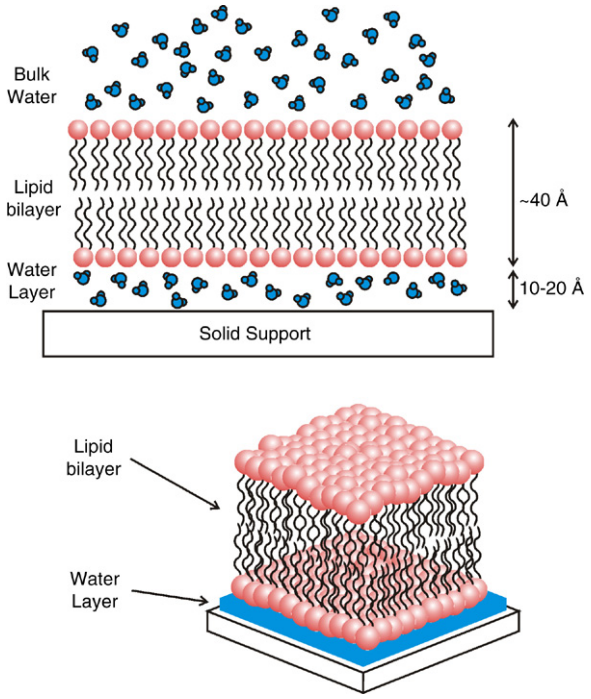

Fig. 4. Schematic diagram of a solid supported phospholipid bilayer. The membrane is separated from the substrate by a 10–20 Å thick layer of water.

However, this also limits the lifetime of the bilayer due to poor stability of the membrane. The methods of detection that can be employed with black lipid membranes are also typically limited. Usually electrical conduction and simple light microscopy are used; however, more recently investigators have begun to utilize more sophisticated optical techniques [52].

3. Solid supported lipid bilayers

Phospholipid bilayers supported by solid substrates are more robust and stable than black lipid membranes. Solid supports also open the door for using surface specific analytical techniques not available for black lipid membranes. In solid supported systems membrane fluidity is maintained by a 10–20 Å layer of trapped water between the substrate and the bilayer [12,60]. A schematic diagram of a supported lipid bilayer is shown in Fig. 4.

The varieties of substrates capable of supporting phospholipid bilayers are somewhat limited. In order to support a high quality membrane (i.e. little or no defects and high lipid mobility) the surface should be hydrophilic, smooth, and clean. The best substrates are fused silica [12,61], borosilicate glass [12, 62], mica [63,64], and oxidized silicon [12]. Attempts have been made to deposit supported bilayers on single crystals of TiO_2 and $SrTiO_2$ as well as on thin films of SiO_2 on $LiNbO_3$ crystals [65–67]. Thin films can be used as solid supports as observed with TiO_2 [68–70], indium-tin-oxide [71,72], gold [73, 74], silver [75], and platinum [76].

There are three general methods for the formation of supported phospholipid bilayers on planar supports for sensor applications. The first method involves the transfer of a lower leaflet of lipids from the air–water interface by the Langmuir–Blodgett technique (Fig. 5a). This is followed by the transfer of an upper leaflet by the Langmuir–Schaefer procedure, which involves horizontally dipping the substrate to create the second layer [12]. A second method of supported bilayer formation is the adsorption and fusion of vesicles from an aqueous suspension to the substrate surface (Fig. 5b) [77, 78]. Also, a combination of the two methods can be employed by first transferring a monolayer via the Langmuir–Blodgett technique followed by vesicle fusion to form the upper layer (Fig. 5c) [79].

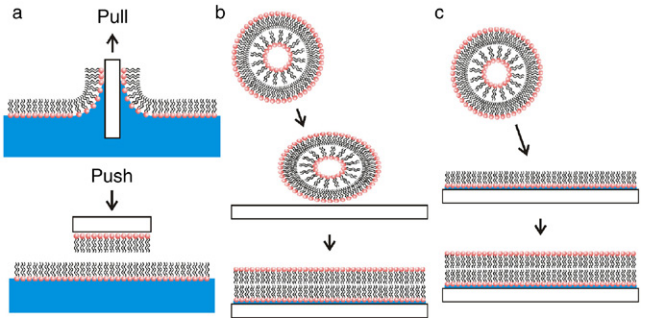

Fig. 5. Common techniques for the formation of supported lipid bilayers. (a) The Langmuir–Blodgett technique is carried out by pulling a hydrophilic substrate through a lipid monolayer and sequentially pushing it horizontally through another lipid monolayer. (b) Vesicles in solution adsorb and spontaneously fuse to the surface to form a solid supported lipid bilayer. (c) A combination of the Langmuir–Blodgett and vesicle fusion processes.

Each of the three deposition methods has its particular advantages and disadvantages. The transfer of amphiphilic molecules from the air–water interface to a solid substrate dates back to the 1920s [80]. An excellent review of this topic is found in Ref. [81]. Tamm and McConnell were the first to apply this technology to form supported phospholipid bilayers by sequential monolayer transfer onto quartz, glass, and oxidized silicon substrates [12]. This method is useful for the formation of asymmetric bilayers [70]; however, it is difficult if not impossible to incorporate transmembrane proteins into the lipid bilayer with this technique because prior to transfer portions of the proteins within the monolayer are exposed to air and can become irreversibly denatured [79].

The adsorption and fusion of small unilaminar vesicles (SUVs) is one of the easiest and most versatile means for forming solid supported phospholipid bilayers (Fig. 5b). SUVs can be prepared by a plethora of methods. The simplest involves the extrusion of multilaminar vesicles through porous polycarbonate membranes at high pressure [82–85]. Another method is the sonication and ultracentrifugation of aqueous lipid suspensions [86]. The incorporation of transmembrane proteins into SUVs requires a gentler process such as detergent removal via dialysis [87,88]. Factors affecting the adsorption and fusion of SUVs to solid supports include: the vesicle composition, size, surface charge, surface roughness, surface cleanliness, solution pH, ionic strength, and the osmotic pressure of the vesicles [68,89]. The process begins with the adsorption of vesicles from the bulk solution onto the substrate (Fig. 6). In the early stages, SUVs may fuse with one another [89]. The vesicles then rupture and fuse to the substrate

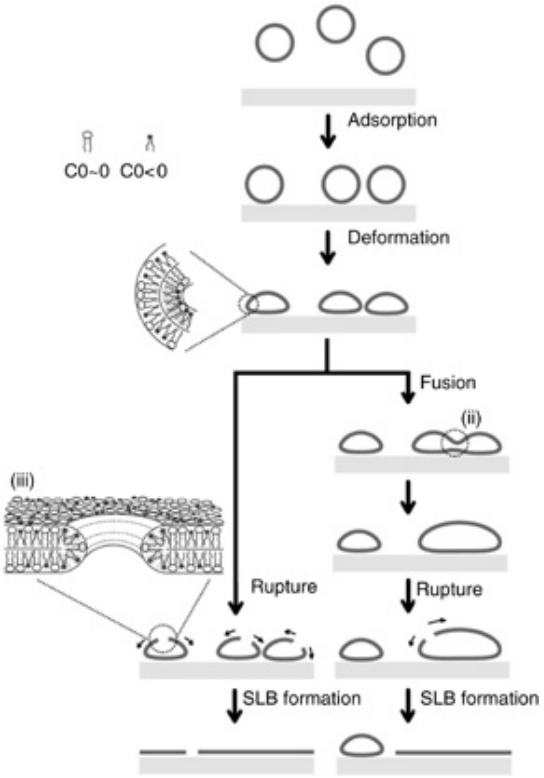

Fig. 6. Proposed method of vesicle fusion. Adsorbed vesicles deform and either rupture or fuse with one another to form larger vesicles which in turn rupture to form a continuous surface supported membrane.
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forming planar supported bilayers in a process that depends upon the chemistry of the individual lipids [90]. The adsorption process can be accelerated by the presence of divalent cations such Ca^{2+} and Mg^{2+} [70]. Fusion of SUVs to the substrate can also be enhanced by heating [72], creating an osmotic gradient across the vesicle membrane [68], and by the addition of fusigenic agents such as polyethylene glycol [37]. Although the exact mechanism of bilayer formation from the adsorption and fusion of SUVs is not fully understood, mathematical modeling of the system has shown good agreement with experimental results [91].

A combination of Langmuir–Blodgett monolayer transfer and vesicle fusion can also be used to form supported phospholipid bilayers [79]. This method involves the fusion of SUVs to a predeposited monolayer of phospholipid. This method is highly efficient for the formation of asymmetric bilayers [92] and for the incorporation of transmembrane proteins into solid supported bilayers [79].

It is well established that phospholipid membranes are held in place above a solid oxide support by a combination of van der Waals, electrostatic, hydration and steric forces [62]. In an egg phosphatidylcholine (egg-PC) bilayer supported on a glass substrate, the underlying water layer effectively lubricates the lipids, which allows them to freely move with a lateral diffusion constant of approximately 1–4 $\mu m^2/s$ [93]. Furthermore, it has been observed that negatively charged vesicles do not easily fuse to glass substrates at basic pH values and low ionic

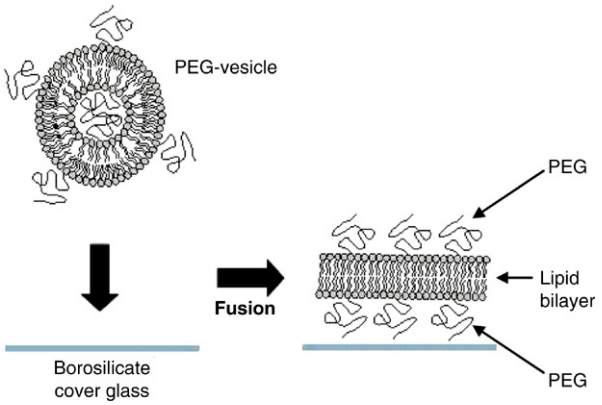

Fig. 7. Illustration of the formation of an air-stable supported bilayer. PEG-PE lipids are incorporated into vesicles which can be fused to solid supports imparting air stability to the system. The PEG layer retains water and increases the bending elastic modulus of the membrane, thus protecting it as it is passed through an air–water interface.
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strengths [62]. Uncharged vesicles made from zwitterionic lipids, however, appear to fuse more readily to Au substrates presenting a charged monolayer rather than to neutral ones [94].

An exciting and newly emerging field in solid supported lipid bilayers is the development of air-stable lipid membranes. Unprotected solid supported lipid bilayers are known to delaminate from the supporting substrate upon passage through an air–water interface [95]. This is problematic when developing practical biosensors based upon supported lipid bilayers because the membrane must be constantly hydrated. It is therefore highly advantageous if the system can be dried after fabrication and rehydrated just prior to use. Systems affording air stability include hybrid bilayers [33], protein stabilized lipid bilayers [95], and polymerized membranes formed using synthetic diacetylene-containing phospholipids [96–99]. However, these systems can suffer from either poor lipid mobility or are almost completely covered with protein. Both of these problems detract from the ability to employ the platform in sensing applications. Recently, an air-stable system has been developed that maintains both high lipid mobility and is capable of binding analyte proteins to ligands presented at the lipid bilayer surface [100]. This is achieved by fusing vesicles containing polyethylene oxide oligomers conjugated to phosphatidylethanolamine (PEG-PE) lipids to glass substrates as illustrated in Fig. 7. The PEG-PE lipids within the bilayer serve two functions. They increase the bending elastic modulus of the membrane and increase the headgroup hydration layer thickness [100]. This combination imparts air stability to the membrane. The PEG layer has also been shown to have a negligible effect on the binding of modest-sized analyte proteins to ligands presented within the lipid bilayer.

Closely related to the air-stable membranes, is the newly emerging area of bilayers which are resistant to cracking upon cooling the bilayer through the gel to liquid crystalline phase transition. Normally, phospholipid bilayers shrink and crack upon cooling [101]. This is potentially a problem for making sensor devices from supported membranes under certain

conditions. Very recently, Granick and coworkers showed that phospholipid bilayers on mica surfaces would resist cracking upon cooling into the gel phase when positively charged lipids were added to dimyristoylphosphatidylcholine vesicles [102]. They argued that these lipids operate by preventing the reorientation of the phosphatidylcholine dipole that normally takes place as the membrane freezes.

The main advantage in using solid supports is clearly an increase in robustness and stability of the phospholipid bilayer membrane. Almost equally important is the ability to probe interactions that occur at the membrane surface with powerful analytical techniques that are surface specific (e.g. atomic force microscopy, quartz crystal microbalance, surface plasmon resonance, vibrational sum frequency spectroscopy, etc.). While solid supported phospholipid bilayers are somewhat limited in terms of their substrate compatibility, their major disadvantage is that the supported membrane is not truly decoupled from the underlying substrate. Indeed, the system may not prevent transmembrane proteins from interacting unfavorably with the underlying substrate. Such interactions with the surface can cause proteins in the membrane to become immobile and hinder their function.

4. SAM/monolayer systems

The use of self-assembly for the modification of electrode surfaces has been the topic of several reviews [103–105]. This includes the use of alkanethiols to form self-assembled monolayers (SAMs) on gold and other widely used electrode surfaces such as silver and mercury. First developed by Nuzzo and Allara at Bell Laboratories in 1983 [106], methyl-terminated alkanethiols on gold provide a well-defined hydrophobic surface to facilitate the formation of a hybrid bilayer membrane [1,32,107]. In its simplest form, the hybrid bilayer membrane consists of a metal supported alkanethiol SAM and a monolayer of phospholipid as illustrated in Fig. 8 [108].

There are a wide variety of alkanethiols which will self-assemble on a gold surface. Octadecanethiol is a typical choice for hybrid bilayer formation due to its ability to form tightly packed and well-ordered monolayers. The SAM layer can be formed by incubating a clean gold substrate with a 1 mM alkanethiol solution in ethanol typically for a minimum of 12 h [108]. Another formation method involves Langmuir–Blodgett transfer [109]. Two general methods exist for applying the phospholipid leaflet to the SAM covered surface: vesicle fusion and lipid transfer from an air–water interface [110]. Vesicles in aqueous buffer have been shown to spontaneously fuse to the hydrophobic surface of supported lipid monolayers [79] and alkanethiol SAMs [32]. The process of vesicle fusion to such surfaces has been investigated by surface plasmon resonance [111], cyclic voltammetry, and impedance spectroscopy [112]. Alternatively, a phospholipid monolayer can be transferred from the air–water interface to the hydrophobic surface of an alkanethiol SAM [113]. This method requires horizontal transfer from a stable phospholipid monolayer supported in a Langmuir trough.

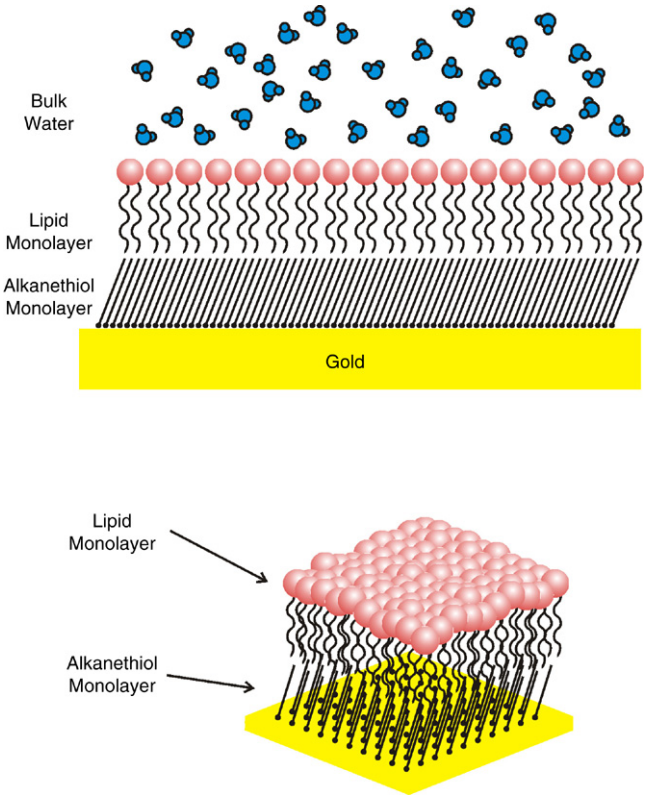

Fig. 8. Schematic illustration of a hybrid bilayer. A single phospholipid monolayer rides on an alkanethiol SAM.

The fusion of ghost cells to alkanethiol SAMs also produces a hybrid bilayer membrane [107,109,111]. Ghost cell fusion offers the ability to reconstitute some of the contents of a cell membrane onto a sensor platform. Such a procedure may eventually represent an efficient means of presenting biomimetic surfaces containing natural mixtures of proteins, lipids, and receptors, as well as cellular membranes from genetically modified cells. It is not clear, however, how transmembrane proteins interact with such surfaces since these species cannot intercalate beyond the alkanethiol SAM.

The physical properties of a hybrid bilayer can be altered through the use of different alkanethiols, lipids, and membrane additives such as sterols and proteins. For example, increasing the chain length of the alkanethiol or phospholipid results in a thicker membrane, thus decreasing its capacitance [32,114]. Altering the composition of the vesicles used to form the lipid layer can also change the properties of hybrid membranes. Incorporation of ligand-conjugated lipids into the membranes is useful for investigations of binding kinetics and multivalent interactions. In this respect, hybrid bilayer formation via the fusion of phospholipid vesicles containing the appropriate ligands has been shown to be effective [1,115].

The underlying SAM layer must only be somewhat modified to accommodate membrane active peptides and transmembrane proteins with small or nonexistent peripheral domains facing the electrode. This can be accomplished through the introduction of ethylene oxide spacer units at the base of the alkanethiol [116]. Examples of proteins that can be investigated in this manner include α-hemolysin

and melittin. These proteins alter the electrical properties of membranes [116], but barely protrude beyond the membrane on the distal side. This has allowed neutron reflectometry investigations [117] to be carried out to determine the orientation of melittin within this lipophilic system. Melittin has also been investigated by cyclic voltammetry in this manner [32,114].

There are several advantages to choosing hybrid phospholipid platforms for sensor applications. Foremost is the coupling of a phospholipid monolayer directly to a metallic surface. This allows for non-labeled analyte detection by direct electrical measurement [33], surface plasmon resonance spectroscopy [1], and quartz crystal microbalance detection [115]. Hybrid phospholipid membranes are often more robust than their solid supported counterparts due to the strong interactions between the alkanethiol SAM layer and the underlying substrate. When formed at an air–water interface, they can be dried and rehydrated while retaining at least some of their original physical and chemical properties [113].

While the rigidity and close packing of the underlying alkanethiol SAM layer provides many advantages, it also presents several limitations. An alkanethiol SAM layer is typically more crystalline in structure [116] than a normal leaflet of a phospholipid bilayer. This results in a less fluid environment. Insertion of proteins is also effected by the packing density of the underlying SAM layer [116]. This can inhibit proper functioning. Of course, transmembrane proteins with both large extracellular and intracellular domains cannot be easily accommodated by hybrid bilayers.

5. Polymer cushioned phospholipid bilayers

While solid supported phospholipid bilayers and hybrid bilayers are excellent sensor platforms for the investigation of many cellular processes, they have difficulty mimicking the appropriate environment for transmembrane proteins, especially those presenting large peripheral domains [118]. The 10–20 Å water layer that resides between a phospholipid bilayer and a solid support provides lubrication and maintains sufficient mobility for the lipid molecules [12,60]; however, the underlying water layer does not protect peripheral portions of transmembrane proteins from immobilization or denaturation if they come in contact with the substrate. Fig. 9 illustrates this problem and also shows the same system in the presence of a lipopolymer support. The desire to properly mimic the inherently complex nature of two-dimensionally fluid plasma membranes has been the driving force for the development of such polymer supported bilayer systems [118].

The addition of a polymer layer effectively decouples the membrane from the surface and still allows for investigation by an array of surface science techniques. In principle, these systems should resist nonspecific adsorption of transmembrane proteins. Another potential advantage of polymeric supports is the ability to avoid nonspecific adsorption of aqueous proteins from solution. Indeed, this can typically occur at defect sites in solid supported bilayers lacking polymer cushions. Large numbers of such defect sites contribute to high background

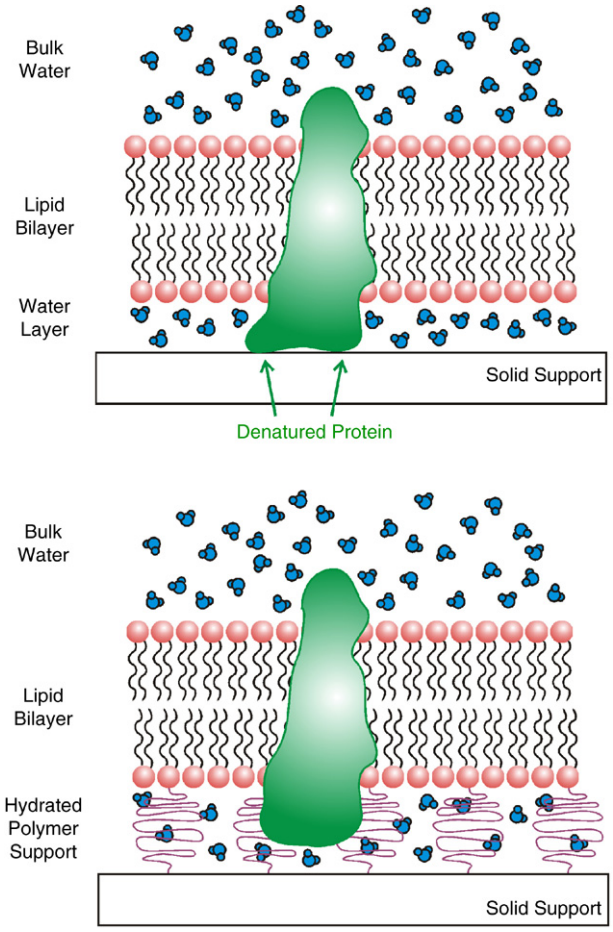

Fig. 9. Peripheral domains of transmembrane proteins can become immobilized and denatured on a solid support. A polymer cushion helps shield the protein from the substrate.

responses and low signal-to-noise ratios especially in electrical detection schemes where electron or ion transport to and from the substrate is monitored [69].

In erythrocyte cells, the cellular membrane is supported by the cytoskeleton, a protein matrix, which supports the lipid bilayer and gives the cell its distinct shape. A well designed polymer cushion should behave much like a cytoskeleton. The design of systems for the support of phospholipid bilayers requires careful consideration of the balancing of surface forces [20]. In physisorbed systems, weak interactions between the phospholipid bilayer and the polymer support can result in an unstable system. This may be overcome by first covalently attaching the polymer layer to the substrate. Next, anchor lipids or alkyl side chains capable of inserting into the phospholipid bilayer are employed. These effectively tether the membrane to the underlying polymer layer. In general, it is desirable for the polymer support to be soft, hydrophilic, not too highly charged, and not extensively cross-linked [20].

There are several types of polymer cushions that have been explored for supporting phospholipid bilayers. These include dextran [23], cellulose [119], chitosan [24], polyelectrolytes [21,120–122], and lipopolymer tethers [19,22,26,27,30,31,123]. Two classes of polymer, polyelectrolytes and lipopolymers, are emerging as popular choices for cushion material. In

the case of polyelectrolyte cushions, the material can be directly adsorbed from solution to a variety of substrates by means of layer-by-layer deposition, providing a great deal of control over the resulting film thickness. Polyethylenimine (PEI) has been used to support phospholipid bilayers on mica [124] and quartz [118,120]. On metallic substrates such as gold, polyelectrolytes can be adsorbed to charged SAMs. Mercaptoundecanoic acid on gold is capable of adsorbing alternating layers of polydiallyldimethylammonium chloride (PDDA) and polystyrene sulfonate sodium salt (PSS) for use as a polymer cushion [21,121].

Polyelectrolyte cushions rely on electrostatic interactions to help hold the system together. Here, alternating charges are the key. Electrostatic attraction between the substrate and polymer cushion binds the polymer layer to the substrate. In turn, van der Waals, hydrogen bonding, as well as electrostatic interactions bond the lipid layer to the polymer. When a polyelectrolyte layer is deposited onto a substrate, charge on the surface builds up repelling additional material with the same charge away from the interface. Under appropriate deposition conditions, a highly uniform film is built with a linear relationship between thickness and the number of adsorbed layers [125]. On the other hand, the necessity of using electrostatic charges to keep polyelectrolyte cushions in place does present certain limitations. Too much charge can adversely affect the function and mobility of membrane constituents and alter interactions between proteins and the supporting cushion. The strength of the attractive forces is also directly affected by the solution environment; namely ionic strength and pH. This can be problematic, as important biological processes occur in different solution environments.

Lipopolymers are another popular class of polymer cushion. They consist of a soft hydrophilic polymer layer presenting lipid-like molecules at their surface which can insert into a phospholipid membrane and tether it to the polymer spacer. Tethering has the advantage of being much less affected by solution conditions such as pH and ionic strength. However, a large degree of tethering can interfere with the mobility of the individual components within the supported membrane [28]. Typically, the lipopolymer is covalently bonded to the substrate. This provides additional support for the membrane system. Attachment of a lipopolymer to a substrate has been carried out via photoreactive coupling [22,23,28], sulfur–metal bond formation [27,29,30], epoxy group linkage [23], or silane bonding [123]. Some common polymer backbones used in the synthesis of lipopolymers are, acrylamide [19,27,30], ethyloxazoline [22,28], peptides [126] and ethyleneglycol [123]. It is important that the polymer cushion have the ability to swell in an aqueous environment and have minimal disruptive interactions with the bilayer and any other reconstituted membrane components [20]. The degree to which a polymer cushion swells in an aqueous or humid environment is a good indication of its ability to be employed as a support. It has been observed that the quality of the supported membrane can also be affected by the degree of swelling of the polymer layer prior to bilayer deposition [118]. Swelling is typically monitored in a home-built humidity chamber and can easily be detected with ellipsometry [23] or surface plasmon resonance spectroscopy [22].

Lipopolymers can be synthesized prior to adsorption onto the substrate or built up on a support by polymer grafting techniques. The lipid tethers are typically attached during polymer synthesis or by reacting specific lipids inside a Langmuir–Blodgett monolayer with active sites on the polymer [26]. Lipids presenting a succinimide headgroup are convenient tethers to link amino groups presented by the polymer support.

Bilayer formation on polymer cushions can be performed by vesicle fusion or Langmuir–Blodgett/Langmuir–Schaffer transfer. The method is basically the same as that shown in Fig. 5. Langmuir–Blodgett transfer of mixed monolayers of phospholipids and lipopolymers from an air–water interface has been shown to provide excellent control over the density of the lipopolymer cushion layer [28,123]. It has also been observed that protein containing vesicles can fuse to these deposited monolayers to form highly oriented transmembrane proteins in the supported system [127]. If the transmembrane protein has peripheral domains that are presented on only one side of the cellular membrane, such domains sometimes prefer to orient into the bulk solution [123].

It should be noted that some polymer supports have been shown to exhibit less than desirable effects on the supported membrane. An imbalance in the stabilization forces or a large number of tethering molecules can decrease the mobility of the supported phospholipid bilayer and alter the phase transition temperature. In some cases polymer supported phospholipid membranes are less stable than those formed directly on an oxide substrate and often possess more defects [20].

Other means of tethering membranes involve the use of ligand–receptor interactions [37] and the direct anchoring of transmembrane proteins to the substrate [128,129] followed by filling in the surrounding lipid film. Ligand–receptor binding of biotin-presenting vesicles to streptavidin monolayers followed by PEG-facilitated vesicle fusion has been suggested to provide a supported bilayer with a protein cushion [37]. Similarly, transmembrane proteins can be anchored to substrates via streptavidin–biotin binding [129] or through the use of His-tag binding to a nickel nitrilotriacetic acid (NTA) complex [128]. Following this anchoring step, a lipid bilayer can be back filled around the suspended transmembrane proteins through *in situ* dialysis, whereby lipid material is deposited and the surfactant required to solubilize the transmembrane proteins is removed [128]. A schematic diagram of this process is shown in Fig. 10. Through genetic engineering, the transmembrane protein can be anchored at precise locations in order to control protein orientation within the membrane.

6. Arrays of supported phospholipid membranes and microfluidic platforms

The use of spatially addressed microarrays presenting large combinatorial libraries of small molecules, DNA, proteins, or peptides is an extremely rapid and powerful means of data collection. The fabrication of such arrays is typically carried

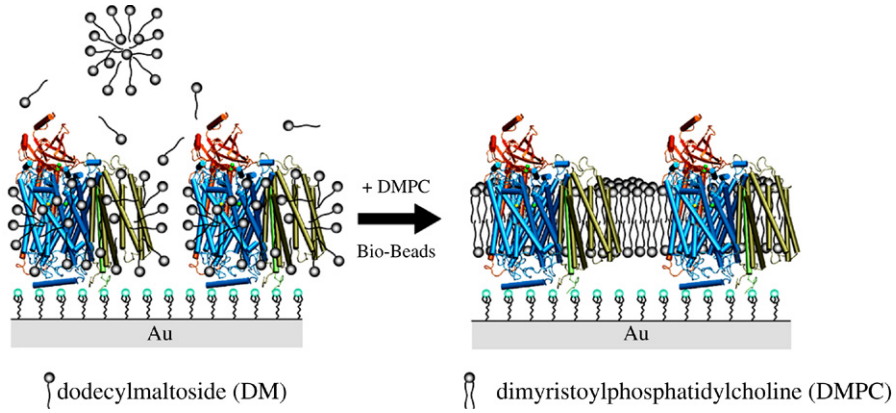

Fig. 10. Illustration of the *in situ* dialysis process for the formation of supported bilayers anchored around transmembrane proteins. The transmembrane proteins are anchored to the surface via the formation of a nickel His-tag complex. Bio-Bead removal of the detergents used to solublize the proteins is carried our simultaneously as the bilayer is filled in by vesicle fusion.
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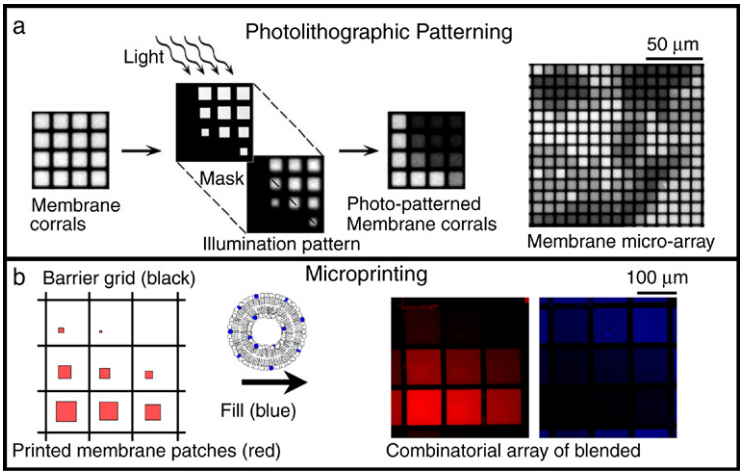

Fig. 11. (a) Composition arrays generated by photopatterning. A mask is used to selectively bleach different sized areas of a membrane array. After diffusive mixing within each corral, a concentration array is observed. (b) Microcontact printing of different sized bilayer patches is used to fabricate a concentration array. After printing, the empty space in each corral is backfilled with SUVs. This forms a continuous bilayer in each corral. Shown here is an epifluorescence image of printed Texas Red labeled membranes backfilled with Cascade Blue labeled lipids. The red image is shown on the right and the blue on the left.
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out by contact printing with a quill pin type printer or by non-contact printing with a piezoelectric print head where small quantities of sample are propelled to the surface. Samples are generally printed from organic solvents onto a reactive surface or one that has a strong nonspecific affinity for the printed molecules. The advantage of such systems is that once a sample is spotted, it can dry on the surface with little or no negative consequences. This is not the case with phospholipid bilayer systems. In order to retain the desired supramolecular structure, a supported phospholipid bilayer must remain hydrated at all times. This creates a significant challenge for fabricating arrays of supported phospholipid bilayers.

In 1997, Groves et al. developed the first method for patterning surfaces with solid supported phospholipid bilayers [13]. A typical formation procedure involved the patterning of photoresist on fused quartz wafers by means of standard photolithographic techniques. SUVs were then fused onto the substrate between the barriers, creating a lithographically patterned array of essentially identical planar supported phospholipid membranes. Each membrane was confined within its own two-dimensional corral. The bilayers retained two-dimensional fluidity within a given corral, but the barriers did not allow mixing between neighboring patches as was demonstrated by fluorescence microscopy [13,130]. Limited differentiation between the bilayers could be achieved by selective photobleaching [131]. Fig. 11a illustrates the process of forming compositional patterns by photobleaching [132].

Barriers to lateral mobility in supported phospholipid bilayers can also be achieved by microcontact printing of proteins onto the substrate prior to SUV fusion or by adsorbing proteins to the solid support after selective areas of a solid supported membrane have been removed by microcontact blotting as shown in Fig. 11b [133,134]. Microcontact printing was originally developed for the patterning of alkane thiols onto gold substrates [135] and involves the use of a stamp such as poly(dimethylsiloxane) (PDMS), that has been molded

against a lithographically patterned surface [136]. The stamp then transfers chemically or biologically relevant materials to a solid substrate.

Kam and Boxer illustrated that corralled membranes can also be made with protein barriers [137]. In this case, cellular adhesion was observed on substrates presenting micropatterned fibronectin and supported phospholipid bilayers. The authors proposed that this system could be useful for the investigation of the responses of anchored cells to stimulants within or presented at the surface of solid supported membranes.

Arrays of supported membranes can also be fabricated by selectively destroying regions of a continuous supported bilayer. This is achieved by high intensity deep-UV illumination through a photomask under aqueous conditions (Fig. 12). The UV radiation generates both ozone and singlet oxygen in highly localized regions. These species react with the lipid to form water soluble components [138]. This method can be exploited to produce patterned phospholipid membranes by backfilling the erased regions with different lipids. It has also been expanded to pattern micron-sized features into bilayers supported on silica microbeads [139].

Another technique for patterning phospholipid bilayer arrays of a single component is the polymer lift off method. Here, a thin layer of Parylene is vapor deposited onto silicon surfaces and patterned to expose the underlying substrate using standard lithographic techniques. After exposure to SUVs, the Parylene layer is peeled away leaving behind patches of lipid bilayers [140–143]. The entire process takes place under water. This method has proven useful for patterning lipid bilayers with a minimum feature size of 1 micron [140] and has been utilized to investigate the effects of receptor clustering [142] as well as ligand–receptor interactions [143].

In addition to simple membrane patterning, spatially addressed arrays of solid supported phospholipid bilayers have also been produced. Spatial addressing enables complete control over the chemical composition of each address in a supported bilayer array. This was first achieved by pipetting from pulled capillaries (Fig. 13) [14]. Additional methods include microcontact printing [134,144,145], laminar flow deposition [18,146], and robotic pin printing [147].

Microcontact printing of composition arrays of phospholipid bilayers was first accomplished by printing different sized bilayers of the same composition into surface patterned corrals. The corrals could then be sequentially backfilled with SUVs containing egg-PC or other chemically relevant species. Fig. 11b illustrates the process of forming composition arrays by microcontact printing [132]. This could potentially be a rapid means of producing arrays which vary in the composition of a few components, although the ability to create multi-component patterns is limited. Another means of microcontact printing bilayer arrays utilizes a stamp fabricated out of agarose [145]. Using this method Majd and Mayer were able to print membrane arrays of one composition at the 200 micron scale and multi-component arrays on the one millimeter scale (Fig. 14). In this case spatial addressing was achieved by hand pipetting different SUV solutions to different regions of the agarose stamp. It may be possible, however, to reduce the size

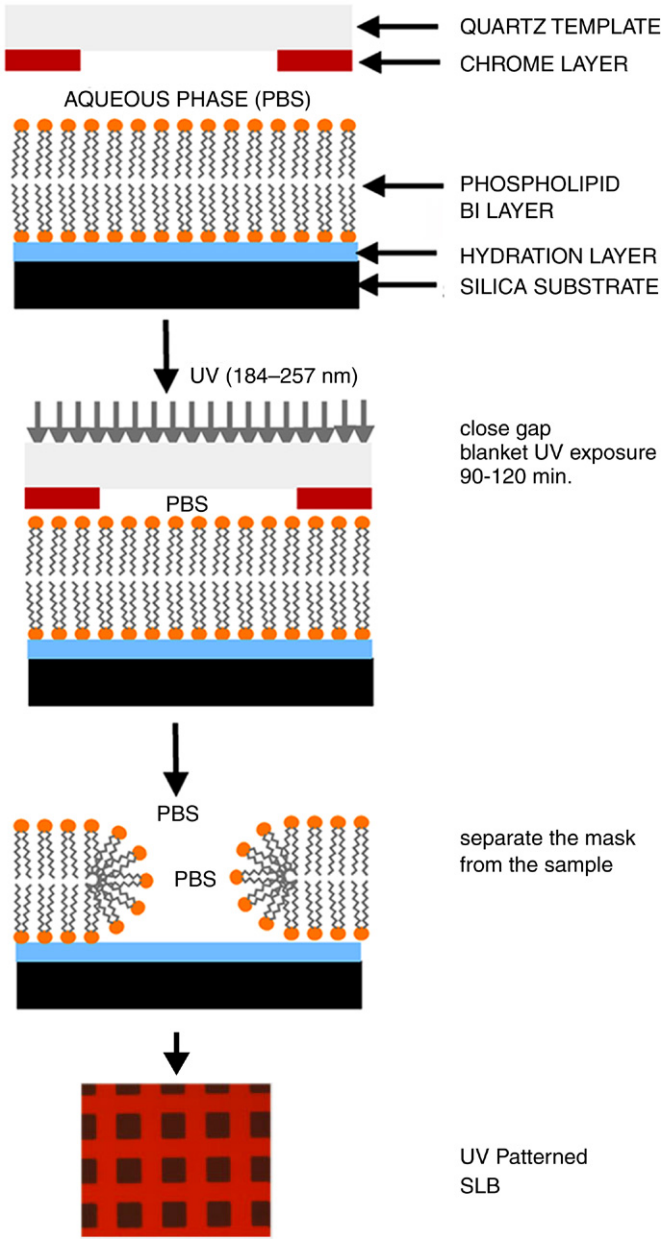

Fig. 12. Depiction of the method for patterning supported bilayers using high intensity deep-UV illumination. Illumination through a photomask in close proximity to a supported bilayer creates localized ozone and singlet oxygen. These highly reactive species decompose the lipids in the regions under illumination. The products of the reaction are soluble and transfer into the bulk solution. This leaves behind a patterned lipid bilayer as can be seen in the fluorescence image.
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of the printed spots if robotic addressing is employed. This method is particularly useful in printing at least 100 copies of the same array with a single inking of the stamp [145].

A more powerful means of addressing phospholipid bilayers is the direct pipetting of SUV solutions into photolithographically patterned arrays [14,15,148]. This method has the advantage that each bilayer can contain any desired composition of lipids or proteins independent of the chemical composition of it neighbors. Fig. 13a illustrates this method for creating spa-

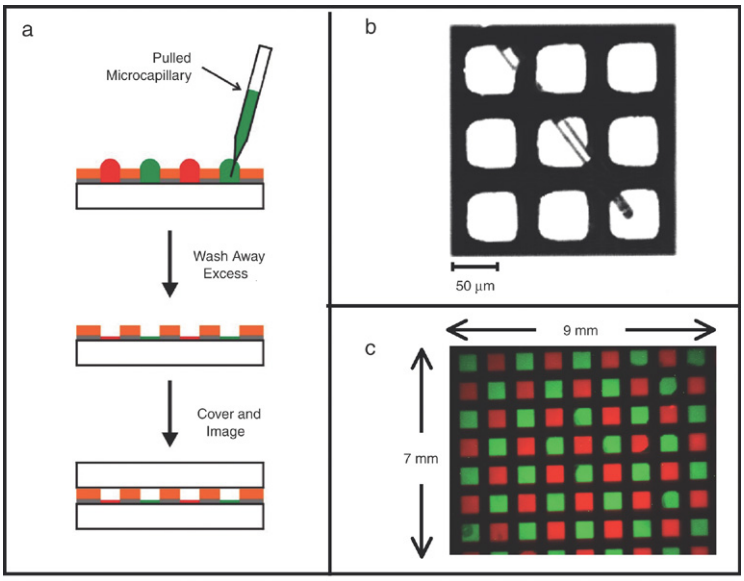

Fig. 13. (a) The spatial addressing of solid supported phospholipid bilayers. A pulled microcapillary tip is used to address individual corrals on a pre-patterned substrate. (b) A bright field image of a pulled microcapillary tip addressing 50 μm corrals. (c) An EFTIR macroscope image of an individually addressed 7 × 9 membrane array. Darker squares have been addressed with Texas Red labeled lipids and the lighter squares with fluorescein labeled lipids.
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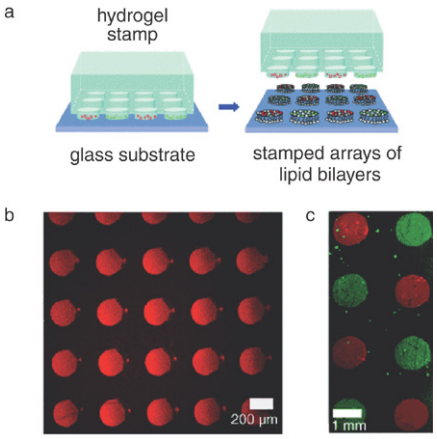

Fig. 14. Arrays of solid supported bilayers stamped with a molded agarose gel. (a) Illustration of the stamping technique. (b) 1 micron supported lipid bilayer patches stamped with a high density array. (c) A low density array demonstrating the spatial addressing capabilities of this technique.
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tially addressed membrane arrays by pipetting of SUVs and Fig. 13b shows a brightfield image of the addressing process. This method has been expanded by employing quill pen printing and robotic addressing in a humidity controlled environment [147].

Due to field of view limitations of fluorescence microscopes, current spatially addressed bilayer arrays have been typically limited to 16 elements or less [14,144,148]. In our laboratory, we have constructed an epifluorescence total internal reflection (EFTIR) macroscope for high numerical aperture, large field of view imaging of such spatially addressed arrays. This allows for an increase in the size and spacing of the corrals thereby easing the difficulty of individually addressing bilayers into a large numbers of distinct locations. Fig. 13c shows the false color image of a 7 × 9 element array of spatially addressed phospholipid bilayers. This method could be further enhanced by the automation of the addressing process or by parallel addressing from an array of tips capable of simultaneously depositing multiple SUV solutions.

The use of laminar flow inside microfluidic channels is also an effective means of producing composition arrays of supported phospholipid bilayers in which two distinct chemical components can be varied simultaneously along a one-dimensional gradient [17,18]. This allows for the addressing of patterned substrates by the flow of concentration gradients of SUVs formed by diffusional mixing of two different SUV solutions [18]. Fig. 15 illustrates the process of forming a one or two component composition array by laminar flow in microfluidic channels [132]. A drawback of this method is the limited number of chemically distinct components that can be simultaneously addressed as well as the lack of control over the ultimate positioning of the bilayers.

A final method of patterning phospholipid membranes on solid supports was achieved by the creation of individually addressable microcompartments above a bilayer array. This was achieved by the microcontact displacement of portions of a continuous solid supported membrane with a patterned PDMS stamp [149]. Yang et al. observed the displacement of membrane regions that came into contact with the stamp. The removal process could be observed in real time by fluorescence microscopy over the course of approximately 90 minutes. Almost all of the displaced phospholipid material formed vesicles in the bulk solution and could be easily rinsed away. The bulk solution in each microcompartment could subsequently be individually addressed. This allowed

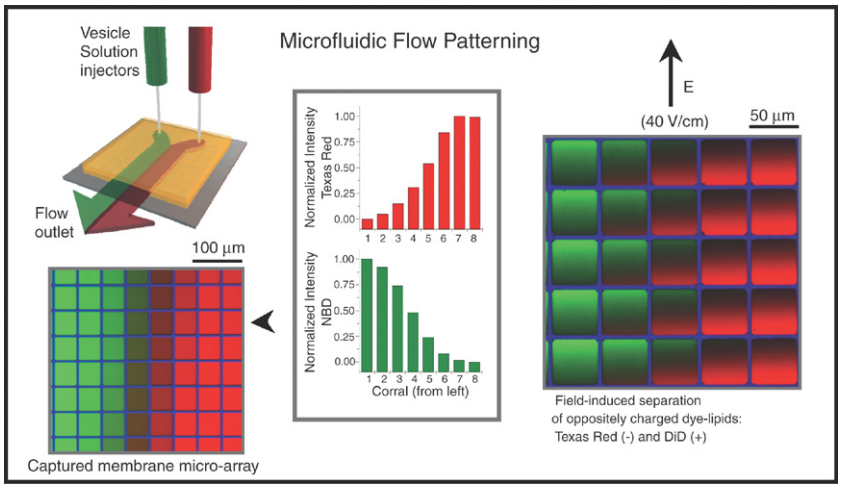

Fig. 15. Addressing by laminar flow in a microfluidic channel. Diffusive mixing in a microchannel under laminar flow conditions provides a concentration gradient of different dye-labeled vesicles. The concentration of vesicles in the gradient is reflected in the surface concentration of each membrane in the resultant array. The array shown is a mixture of Texas Red labeled lipids (shown in red) and DiD labeled lipids (shown in green). Since the dyes have opposite charge, they can be separated in an electric field.
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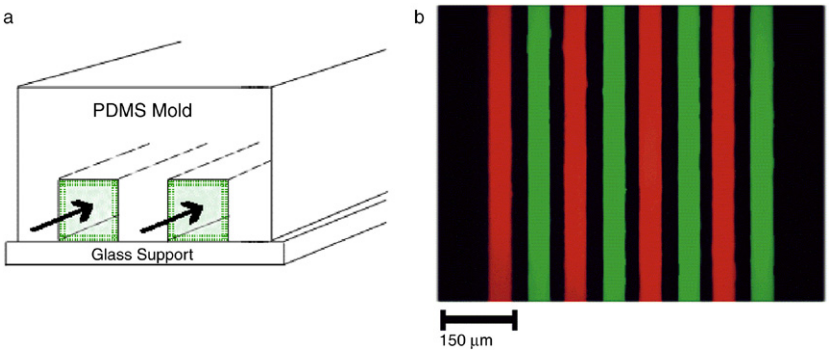

Fig. 16. (a) Schematic diagram showing phospholipid bilayers coating the interior of PDMS-glass microchannels. (b) Epifluorescence image of the system.
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for the study of multiple ligand–receptor binding inhibitors to be carried out in a parallel fashion at the membrane/solution interface on a single chip. Such experiments may ultimately prove useful for the creation of high throughput drug discovery assays.

7. Bilayer coated microfluidics

The union of microfluidics and solid supported phospholipid bilayers has provided a powerful analytical platform for the investigation of multivalent ligand–receptor binding. PDMS/glass microfluidic systems [136] offer an inexpensive and simple sensor platform in which to perform analytical measurements. SUVs injected into the microchannels have been observed to form a supported phospholipid bilayer coating when the polymer surface was rendered hydrophilic in an oxygen plasma [3]. Fig. 16a illustrates the concept of bilayer formation inside PDMS microchannels and Fig. 16b shows a fluorescence image from dye-labeled phospholipids within these bilayers. This technology has been utilized to perform on-chip immunoassays within microfluidic channels. Yang et al. were able to measure the binding constant between fluorescently labeled bivalent antibodies and hapten-presenting phospholipid bilayers with unprecedented signal-to-noise and only minute quantities of protein [3]. They were also able to investigate the effects of hapten density on the binding process [4].

The employment of phospholipid bilayers inside microfluidic channels allows for the rapid determination of enzyme kinetics by linking these catalysts to the bilayer [16]. Immobilizing enzymes on the surface of phospholipid bilayers has the advantage of protecting the proteins from denaturation on the walls of the device. This insures that the maximum amount of enzyme remains active throughout a given experiment. Mao et al. illustrated that it is possible to perform one-shot Lineweaver–Burk analysis using alkaline phosphatase enzymes bound to supported lipid bilayers via biotin–streptavidin linkages [16]. This method of performing enzyme assays required only small amounts of protein and provides data with a greater signal-to-noise ratio than traditional techniques.

Microfluidics in conjunction with a linear temperature gradient has been utilized to measure the phase transition temperature of a solid supported phospholipid bilayer [150]. The generation of linear temperature gradients inside microfluidic devices

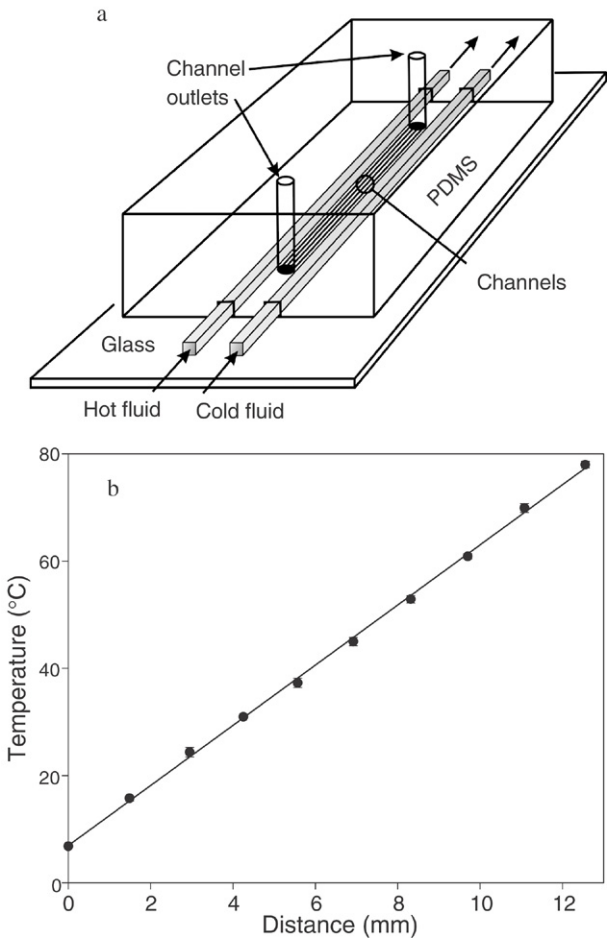

Fig. 17. (a) Schematic diagram of a microfluidic device used to measure the phase transition temperature of a DPPC bilayer. (b) Calibration curve showing the temperature at various positions within the device.
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is possible due to the short length scales of the microchannels. Mao et al. first demonstrated that a linear temperature gradient can be established across an array of microchannels between a hot source and a cold sink (Fig. 17) [150]. The use of multiple microchannels adds structural support to the system and eliminates the effects of mixing by convection. By correlating each channel to its respective temperature and acquiring data from all channels simultaneously, it was possible to measure the phase transition temperature of a dipalmitoylphosphatidylcholine (DPPC) bilayer. The microfluidic temperature gradient has numerous other applications ranging from the optimization of chemical and biochemical synthesis to measuring the activation energies of immobilized enzymes within the microchannels [150].

8. Supported lipid bilayers and nanotechnology

A significant application of phospholipids in nanotechnology is the creation of networks of connected surface-immobilized vesicles (Fig. 18). These networks are referred to

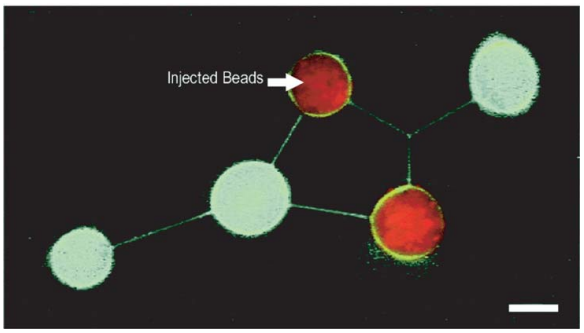

Fig. 18. Epifluorescence image of an NVN stained with a green dye. The red regions show where 30 nm latex beads have been injected into the system. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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as nanotube vesicle networks (NVNs) [40]. NVNs can be created by three methods; mechanical fission [151], micropipet-assisted formation [152], and micropipet writing [153]. The use of mechanical fission to form NVNs offers the least control over vesicle size and is limited to systems that are not geometrically complicated. It involves the use of a small carbon fiber that has been coated with bovine serum album (BSA) to separate a surface immobilized giant unilaminar vesicle (GUV) into two vesicles through homofission by depressing the carbon fiber through the GUV [151]. The two vesicles, which are connected by a nanotube of phospholipid material from the vesicles, can then be separated by a lateral motion of the carbon fiber. Separation distances of a few hundred microns can be easily achieved [151].

The formation of NVNs by micropipet-assisted formation requires the use of applied DC voltage pulses between a tapered micropipet tip and a carbon reference electrode [152]. Penetration and removal of a GUV by the micropipet tip results in phospholipid nanotube formation when performed in aqueous solution. Applying pressure with the micropipet tip inflates a vesicle on the end of the nanotube, which can then be placed at the desired location. This process can be repeated several times, constructing a complex network. The last vesicle is removed from the tip by application of mechanical force and applied DC voltage pulses. This method can also be used to inject materials into the NVN. To do this, the micropipet tip is filled with the solution that is to be injected into the NVN and used to form a vesicle connected by a nanotube to the network. If this vesicle is released into solution instead of being immobilized first, it will be drawn back by surface tension and release its contents within the NVN [154].

Micropipet writing of NVNs can be achieved through the use of micropatterned gold discs that have been functionalized to present neutravidin [153]. First, a GUV presenting biotin at its surface is suctioned into a micropipet tip. Next a small vesicle is formed at the tip of the micropipet by applying a positive pressure. Finally, this vesicle is brought into contact with one of the micropatterned gold circles. The ligand–receptor interaction of neutravidin and biotin is strong enough to hold

the vesicle in place while the micropipet is translated away from the first vesicle, drawing with it a phospholipid nanotube. The process of vesicle formation and immobilization can be repeated several times until the lipid material inside the micropipet is exhausted, creating a complex network on the micropatterned surface [153].

By applying a positive pressure to one of the vesicle compartments, the transport of individual fluorescent beads through the nanotubes from one compartment to the next has been observed [151]. NVN technology has also been applied to investigate exocytosis [155], a process in which small vesicles fuse to the outer membrane of a cell and release their contents, as in neural communication. Also, enzymatic reactions have been studied inside NVNs employing as few as 15 enzymes [156]. Eventually, NVNs may prove useful for monitoring stochastic processes at the single molecule level.

9. Future directions

In the future, substrate supported phospholipid bilayer technology will continue to play a pivotal role in the development of sensors and nanodevices. If developed to a commercial level, arrays of supported phospholipid bilayers have the potential to accelerate research in the field of proteomics just as DNA microarrays accelerated genomics research. Such a possibility is quite important as approximately half of all known drug targets are membrane associated proteins [157]. Combining microfluidic platforms and array-based systems with current advances in solid supports for phospholipid membranes should enable a multitude of sensing applications. For example, these platforms could provide valuable information for the development of early warning sensors for biological warfare agents, the discovery of novel drugs, and therapeutic methods for combating, not only viral diseases such as AIDS, influenza and severe acute respiratory syndrome (SARS), but also neural degenerative diseases from Alzheimer's to Parkinson's.

Perhaps the ultimate accomplishments in phospholipid membrane nanotechnology will be in the field of medicine. By combining nanofluidics, encapsulated enzyme technology, and protein engineering, transport could be facilitated into and out of a set of bioreactors in a series or parallel fashion. With gated channels and molecular specificity, one could construct powerful synthetic machinery. Such platforms could be used as artificial cells or miniature factories. One could even envision these systems being used as therapeutic agents. By presenting the proper ligands and proteins at the surface of a supported membrane, phospholipid bilayer-enveloped nanodevices could be directed to a specific location such as a tumor cell. Once in place, they might control the delivery of chemotherapies as well as manufacture them *in vivo*.

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