

Review

Supported planar bilayers in studies on immune cell adhesion and communication

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Abstract

Supported planar bilayers have been used extensively in immunology to study molecular interactions at interfaces as a model for cell–cell interaction. Examples include Fc receptor-mediated adhesion and signaling and formation of the immunological synapse between T cells and antigen-presenting cells. The advantage of the supported planar bilayer system is control of the bilayer composition and the optical advantages of imaging the cell–bilayer or bilayer–bilayer interface by various types of trans-, epi- and total internal reflection illumination. Supported planar bilayers are simple to form by liposome fusion and recent advances in micro- and nanotechnology greatly extend the power of supported bilayers to address key questions in immunology and cell biology.

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1. General introduction

Supported planar bilayer technology has developed and evolved in parallel with the molecular era of immunology and has been involved in a number of decisive experiments in understanding cell–cell communication in the immune response. Early application of planar bilayer technologies to immunology employed black lipid bilayers, a technology developed by electrophysiologists in which the bilayer is

suspended over an aperture and both sides are accessible (Henkart and Blumenthal, 1975; Wolf et al., 1977), or artificial target cells, in which a lipid bilayer is formed on the surface of a porous bead (Takai et al., 1987). The general application of artificial bilayer technology to immunology was expanded by the introduction of glass-supported planar bilayers by McConnell et al. in the early 1980s (Hafeman et al., 1981; Balakrishnan et al., 1982). The simplest form consists of a substrate like glass, quartz or mica, on which a phospholipid bilayer is deposited either one leaflet at a time from an air–water interface or by fusion of preformed liposomes (McConnell et al., 1986). The structure of the supported planar bilayers will be discussed in more detail later, but the key

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features are that the individual lipid molecules are highly mobile and anything linked to the phospholipids or glycolipids in the upper leaflet retains this mobility. Supported planar bilayers have evolved into a platform for studies on molecular patterning, and the planar bilayer technology is poised to play an important role in the interface of nanotechnology and biological systems (Sackmann, 1996; Dustin et al., 1998; Groves and Boxer, 2002). Here, we will review applications of planar bilayer technology to the immune system and discuss current directions in research that point to novel capabilities of the technology and provide examples of pressing biological questions that can be addressed with these tools.

2. Supported bilayers in study of immune cell communication: Fc receptor systems

Soon after the realization that glass-supported planar bilayers can be generated by deposition of lipid monolayers on a clean glass surface, the system was applied to immunologically important topics. Fc receptor function can be reconstituted through a very simple and elegant system using phospholipids with head groups that can be bound by high-affinity antibodies. These phospholipids include natural products such as cardiolipin and artificial derivatized phospholipids such as dinitrophenol or fluorescein groups added to free amino group of phosphatidylethanolamine (Humphries and McConnell, 1975). Antibodies to these phospholipids can then be attached to the bilayer leaving the constant region (Fc) fully accessible to receptors on the surface of cells that settle on the bilayer (Hafeman et al., 1981). The accessibility of the head group to the antibody was dependent upon the structure of the bilayer and can also be modified by changing the spacing of the head group from the glycerol backbone of the phospholipid (Balakrishnan et al., 1982). This approach to putting proteins of interest on the bilayer avoids many early pitfalls related to the incompatibility of proteins with organic solvents used in preparation of lipid monolayers, an intermediate in the original procedure for supported bilayer formation. Antibodies attached to the bilayer have exposed Fc regions that are engaged by Fc receptors (FcR) on the live cell. Depending upon the isotype of the antibody, different FcR are engaged

with different functional effects. Interactions of Fc γ R with IgG on the bilayer trigger adhesion. Interaction of Fc ϵ R with IgE on the bilayer triggers degranulation of mast cells.

3. Minimal requirements for antigen presentation

Supported planar bilayers were used to demonstrate that MHC–peptide complexes are the physiological ligand for the T cell receptor (TCR). MHC classes I and II antigens are type I transmembrane proteins that were readily purified by immunoaffinity chromatography from antigen-presenting cells. A breakthrough in incorporating these proteins into supported planar bilayers was the use of liposome fusion (Brian and McConnell, 1984), rather than monolayer deposition. Transmembrane proteins are readily incorporated into small unilamellar liposomes by detergent dialysis (Mimms et al., 1981). These liposomes were found to fuse to clean glass surfaces to form supported bilayers that are very similar to those formed by monolayer deposition. Brian and McConnell (1984) reconstituted allogeneic MHC class I molecules in supported planar bilayers and found that they could be used to activate CD8 positive T cells, and Watts et al. (1984) had similar findings with allogeneic MHC class II molecules activating CD4 positive T cells. Liposomes with allogeneic MHC class I added directly to cultures with T cells could also elicit responses (Herrmann et al., 1982), but it is likely that the active form of these liposomes was either as a supported bilayer or plastic-bound liposomes since subsequent studies determined that small unilamellar liposomes are not large enough to directly activate T cells (Mescher, 1992). It was not clear in the early 1980s how the allogeneic MHC class I and II molecules functioned. Unanue (1984) had proposed years earlier that protein antigen was broken down to peptides that associate with MHC molecules to form antigenic structures. Babbitt et al. (1985) were able to demonstrate with a syngenic MHC that short peptides derived from the foreign antigen, once bound to the MHC class II molecules, formed a ligand for the TCR. This finding provided a mechanism for the earliest findings on MHC restriction (Zinkernagel and Doherty, 1974). The MHC–peptide complex was visualized crystallographically several years later

revealing a structural basis for peptide interaction and presentation by MHC molecules (Bjorkman et al., 1987; Fremont et al., 1992). Subsequently, it was found that alloantigenic MHC contains a subset of specific peptides that together with the allogeneic MHC can generate very high-affinity ligands for a high proportion (~ 10%) of TCR in a mismatched recipient (Heath et al., 1991). While most studies used planar-supported bilayers formed on glass coverslips, bilayers supported on glass beads were also effective in activating T cells (Gay et al., 1986). In a preview of technologies that are only now becoming widely available, Watts et al. (1986) used total internal reflection illumination to demonstrate the TCR-induced association of a fluorescent peptide with MHC molecules labeled with a different fluorescent dye. While this mode of peptide association with MHC may not be the common mode in biological systems where the MHC–peptide complex is formed within the cell and is relatively stable (Cresswell, 1994), it is possible that unstable MHC–peptide complexes formed at the cell surface may be important in some situations, particularly in autoimmune states (Pu et al., 2002). All the studies on planar bilayers measured population parameters like IL-2 production from T cells or proliferation and ignored the physical interaction of the T cells with the supported bilayers. It was assumed that the cell interacted and, in retrospect, the adhesion molecules that mediated this interaction were probably supplied by serum proteins adsorbed to the phospholipid bilayers, which are far from being biologically inert (Bonte and Juliano, 1986). A major focus of studies in the last 15 years has been to understand this physical interaction and its requirements.

4. Supported bilayers in analysis of cell adhesion

The study of the physical interaction of cells with supported planar bilayers was inspired by the aforementioned antigen presentation studies and sought to better understand the T cell–APC communication interface by simplifying it. A large number of adhesion molecules were identified by monoclonal antibodies and were readily purified in active form. In the mid-1980s, the mechanism of cell adhesion was still controversial and clear examples of receptor interac-

tions that mediated cell attachment were limited. One of the first cell adhesion systems to be defined and reconstituted was based on the interaction of CD2 and CD58. Monoclonal antibody inhibition studies pointed to an adhesion mechanism based on the interaction of CD2 (also called T11, sheep red blood cell receptor and LFA-2) with CD58 (also called T11 target structure and LFA-3) (Hünig, 1985; Shaw et al., 1986; Vollger et al., 1987). CD2–CD58 interactions were directly demonstrated using purified CD2 and purified CD58, the later reconstituted in supported planar bilayers (Dustin et al., 1987a; Selvaraj et al., 1987). The CD2–CD58 interaction was also demonstrated using artificial target cells, bilayers supported on hydrophobic polymer beads (Takai et al., 1987). T cell adhesion to bilayers bearing CD58 was remarkably efficient, but was not accompanied by the cell spreading that characterized adhesion to planar bilayers containing ICAM-1 (Marlin and Springer, 1987; Dustin et al., 1988; Dustin and Springer, 1988). These studies were the first to truly define unambiguous ligand–receptor pairs that mediate the physical attachment of cells to each other.

5. Changes in technology that allow direct detection of receptor interactions in the cell–bilayer interface

In parallel with the initial adhesion studies, it was found that CD58 existed in both glycosylphosphatidylinositol (GPI)-anchored and transmembrane forms (Dustin et al., 1987b). This naturally arising situation provided an experimental opportunity to test the role of ligand lateral mobility in adhesion based on exploiting the properties of the supported planar bilayer system. It is generally found that transmembrane proteins are laterally immobile in the supported planar bilayers, perhaps because the cytoplasmic domains are trapped between the bilayer and the glass support (Salafsky et al., 1996). In contrast, it was anticipated that the GPI-linked proteins would be laterally mobile because they only interact with the upper leaflet of the bilayer (McConnell et al., 1986). Consistent with these predictions, it was found that the GPI-anchored form of CD58 was highly mobile in the supported planar bilayers, whereas the transmembrane form of CD58 was completely immobile (Chan et al., 1991). The mobility of directly labeled GPI-

anchored proteins in the bilayer is in the range of 40% to greater than 95%. The basis of the immobile fraction of GPI-anchored proteins, when present, is not clear, but may include defects in the bilayer or the trapping of some proteins between the bilayer and glass. The mobility of a given molecule can also be density-dependent. For example, CD16 is highly mobile up to 3000 molecules/ μm^2 , but bilayers prepared at 4000 molecules/ μm^2 showed no mobility, apparently because the high protein density interfered with planar bilayer formation. Dilution of the liposomes with protein-free liposomes restored mobility and resulted in a linear reduction in density as a function of the mixture of protein-containing and protein-free liposomes. This is technically useful since a single high-density liposome preparation can be mixed with other liposomes to form bilayers with predictable densities of single or multiple molecules. The physiological density range for most adhesion molecules would be up to 1000 molecules/ μm^2 so the physiological range of expression levels can generally be explored.

The behavior of GPI-anchored and transmembrane forms of CD58 in biological membranes may be quite different, but nonetheless, the experimental system of the planar bilayer offered a clean system to investigate effects of ligand lateral mobility. The laterally mobile form of CD58 was 50-fold more potent than the immobile form of CD58 and stabilized adhesion faster (Chan et al., 1991; Tözere et al., 1992). One mechanism by which the GPI-anchored form could have an advantage in adhesion is through the accumulation of mobile ligands in the interface between the cell and bilayer. Mobile ligands accumulate at the interface between beads coated with ligands and cells coated with high-affinity antibodies to these ligands (McCloskey and Poo, 1986). Receptor–ligand interactions in the interface deplete free ligand and generate concentration gradients, which cause more free ligand to diffuse into the contact area. Ultimately, an equilibrium can be reached in which bond formation and bond dissociation are balanced and the total amount of ligand (bound and free) in the contact area is enriched. Labeling of CD58 with a fluorescent dye allowed the visualization of interactions between cell surface CD2 and fluorescent CD58 in the planar bilayers (Dustin et al., 1996a). The CD2–CD58 interaction has a very low affinity and fast off-rate

in solution (van der Merwe et al., 1994), and this is reflected in the dynamics of interactions in contact areas (Dustin, 1997; Dustin et al., 1997b). Using the supported planar bilayer technology, it is possible to measure 2D affinity and kinetic rates in contact areas—allowing a quantitative basis for understanding interactions in contact areas (Dustin et al., 2001). Initially, it was approximated that the density of free ligands inside the contact area was equal to that surrounding the contact area (Dustin et al., 1996b). However, the use of labeled molecules in the bilayer that did not interact with cellular receptors revealed that the density of free molecules in the contact area is less than in the surrounding bilayer (Bromley et al., 2001). This exclusion may be due to the space occupied by the cellular glycocalyx in the contact area and is around 20% for contacts mediated by human CD28–CD80 interactions when examined with labeled mouse CD48, which does not interact with receptors on human T cells. A consequence of this exclusion is that at very high ligand densities, the contact areas can appear as a dark area in terms of total fluorescence, even when interactions are present. This exclusion effect can be quantitatively accounted for to allow accurate measurements over the entire range of ligand densities (Bromley et al., 2001). The planar bilayer system allows these quantitative measurements of interactions to be taken to a new level. Other types of analysis can also be applied to bilayer interactions including application of surface force microscopy (Leckband et al., 1995) and interference reflection microscopy (Albersdorfer et al., 1997).

6. Supported bilayers in studies on molecular patterning in cell–bilayer contact areas

Early structural information on T cell adhesion and signaling receptors suggested that cell–cell communication would naturally require molecular segregation based on the different size of adhesion molecules (Springer, 1990). This concept has been incorporated into models for signaling based on the idea that negative regulatory molecules with large extracellular domains can be excluded from close contacts containing signaling TCR and costimulatory molecules to initiate or sustain signaling (Davis and van der Merwe, 1996; Shaw and Dustin, 1997). Direct evi-

dence for molecular segregation in contact areas was obtained using both cell–bilayer and cell–cell interaction experiments (Dustin et al., 1998; Monks et al., 1998). Segregation based on both passive (Dustin et al., 1998) and active (Dustin et al., 1998; Monks et al., 1998) cellular processes was demonstrated. Structural information on integrins and immunoglobulin superfamily members suggest that the LFA-1–ICAM-1 adhesion molecule pair may span a distance of ~ 40 nm between apposed membranes, whereas the Ig superfamily members CD2 and CD58 and the TCR–MHC–peptide interaction span a distance of ~ 15 nm. It was found in cell–cell systems that TCR–MHC–peptide enriched regions are segregated from LFA-1–ICAM-1 enriched regions of aldehyde-fixed T cell–B cell contact areas. Furthermore, the molecules are not only segregated, but further organized into concentric structures with TCR–MHC–peptide accumulation in the central supramolecular activation cluster (cSMAC) and LFA-1–ICAM-1 accumulation in a ring-like peripheral supramolecular activation cluster (pSMAC) (Monks et al., 1998). Planar bilayer studies concurrently demonstrated spontaneous segregation of CD2–CD58 interactions from LFA-1–ICAM-1 interactions. In these studies, the CD58 and ICAM-1 were both GPI-anchored and therefore mobile in the supported planar bilayer. In the absence of T cell activation or the cytoplasmic domain of CD2, the segregated patterns were generally disorganized (Dustin et al., 1998). T cell activation resulted in induced interactions of CD2 with CD2AP, leading to higher order organization of the segregated domains into an “immunological synapse” (Dustin et al., 1998). The immunological synapse was defined as a stable junction between immune cells in which adhesion molecules are segregated from antigen receptors and the cells display specific polarization of cytoskeletal and secretory structures. The stable T cell–APC interface has been broadly defined as an immunological synapse and the subdomains defined as SMACs.

The dynamics of immunological synapse formation has been the subject of studies with cell-supported planar bilayers and cell–cell systems. The continuing complementarities of the bilayer and cell–cell systems are partly based on the superior resolution and simplicity of interpretation of bilayer-based images, balanced by the caveats of replacing a live cell with an

artificial substrate. The dynamics of immunological synapse formation follow similar patterns in cell–bilayer and cell–cell systems. Generally, TCR–MHC interactions are initiated in peripheral regions of the contact, which take the form of a ring in a more ordered bilayer system or microclusters in the cell–cell system (Grakoui et al., 1999; Krummel et al., 2000). Grakoui et al. defined this stage as a nascent or immature immunological synapse (Fig. 1). This ring or peripheral microcluster pattern then inverts resulting in a TCR–MHC cSMAC and an LFA-1–ICAM-1 pSMAC. This stage is defined as a mature immunological synapse and may remain stable for several hours (Grakoui et al., 1999; Krummel et al., 2000). The movements of other molecular components in the immunological synapse have been reported including CD4, an MHC class II binding protein that co-clusters with TCR in the nascent immunological synapse, but appears to localize to the pSMAC in the mature immunological synapse. The large phosphatase CD45 has also been extensively studied since it is implicated in positive and negative regulation in early TCR signaling. CD45 is excluded early (Johnson et al., 2000; Leupin et al., 2000), but a significant amount of CD45 returns to the cSMAC, apparently in an endosomal vesicle population (Johnson et al., 2000). Freiberg et al. (2002) found that TCR and CD45 are colocalized in cSMAC-like structures in

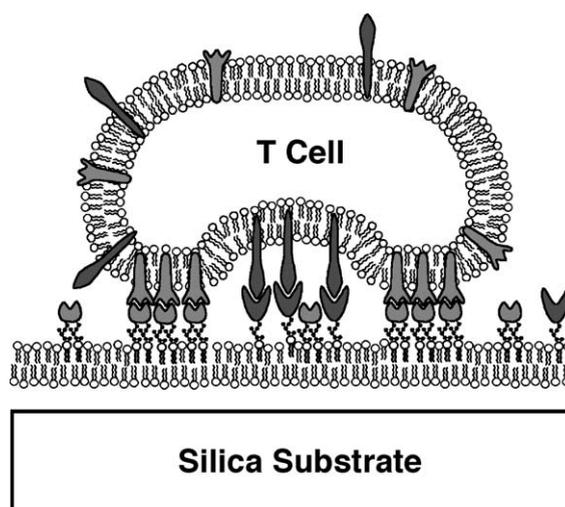


Fig. 1. Schematic of nascent immunological synapse. The planar bilayer contains ICAM-1 (dark gray) and MHC–peptide complexes (light gray) that interact with LFA-1 and the T cell receptor, respectively, on the T cell surface.

the first minute of contact formation between the T cell and APC, but are then segregated to allow robust early signaling. These early events have not been addressed in the bilayer system, but the experience with the bilayer system suggests that the early TCR/CD45 colocalization is likely to be in membrane invaginations or endosomal compartments. The costimulatory molecule CD28 has also been studied due to its important role in primary T cell responses. CD28 is distinguished from adhesion molecules like CD2 by relatively low expression and low lateral mobility CD28 on naive murine T cells. These factors make CD28–ligand interactions highly dependent upon antigen receptor signaling. Therefore, while CD28–ligand interactions are similar to many adhesion mechanisms, the interaction has no effect on TCR–MHC–peptide interactions, unlike adhesion molecules, and appears to be more important for signaling when engaged in parallel with the TCR (Wülfing et al., 1998, 2002; Bromley et al., 2001). Therefore, a number of molecules that are important for T cell activation are integrated into the immunological synapse. The supported planar bilayer system has provided an accurate and very clear view of this process based on continual cross-checking with cell–cell systems.

7. Methods for formation of supported bilayers

A variety of methods of forming supported planar bilayers have been developed. The most general is vesicle fusion, which was originally introduced by Brian and McConnell (1984) and further developed by others (Groves and Boxer, 1995; Salafsky et al., 1996). In this process, unilamellar vesicles adsorb, rupture, and ultimately fuse together to form a single and continuous phospholipid bilayer on a solid substrate. Vesicle fusion can also be used to form supported bilayers on silica microbeads as small as 1 μm in diameter (Bayerl and Bloom, 1990). Other processes of supported bilayer deposition include monolayer transfer from the air–water interface (Tamm and McConnell, 1985), which assembles the bilayer one leaflet at a time, bilayer spreading from a lipid reservoir (Rädler et al., 1995), and a newly introduced technique that involves assembly of proteins and lipids onto the substrate directly from a detergent

solution (Karlsson and Löfås, 2002). Irrespective of the method of assembly, interactions between bilayers and solid substrates such as silica and some polymers involve electrostatic and hydration forces, both of which can be repulsive, and van der Waals forces, which are strictly attractive. This combination of physical forces creates an energetic minimum that tightly traps the bilayer near the surface (Sackmann, 1996; Cremer and Boxer, 1999; Sackmann and Tanaka, 2000). Covalent tethering of the bilayer to the solid substrate has also been employed as an additional mechanism of establishing adhesion (Raguse et al., 1998). Specific interactions between the bilayer and the substrate are advantageous when bilayer assembly on materials, such as gold, that do not favor the vesicle fusion method is required. However, the fluidity of such tethered bilayers can be a concern. Bilayers formed by vesicle fusion on silica are the best characterized and most widely used in studies of cell–cell interactions.

A critical and enabling feature of silica-supported lipid bilayers is the free lateral diffusion of lipids and lipid-linked proteins over macroscopic distances on the bilayer surface. The bilayer is separated from the silica substrate by a thin (~ 10 Å) layer of water, which preserves the intrinsic fluidity as well as the bilayer structure (Bayerl and Bloom, 1990; Johnson et al., 1991; Koenig et al., 1996). Lipids and lipid-linked proteins such as the GPI-linked proteins enjoy uninterrupted lateral diffusion in supported bilayers. Transmembrane proteins tend to be immobilized, presumably due to anchoring to the underlying substrate, while remaining otherwise functional. Supported bilayers have been formed on a variety of polymeric substrates in an effort to afford greater control over the mobility of transmembrane proteins (Kühner et al., 1994; Wong et al., 1999a,b; Sackmann and Tanaka, 2000). Although this is a promising strategy, bilayers on polymeric substrates are substantially more difficult to work with and, to date, have not been applied to problems in cell biology as widely as glass-supported bilayers.

Phospholipid disorder facilitates the vesicle fusion process itself. Continuous supported bilayers are best formed at temperatures above the melting temperature of the acyl chains. The natural lipid extract, egg phosphatidylcholine, is quite fluid at room temperature, due largely to a high percentage of unsaturated

phospholipids. For lipids such as dimyristoylphosphatidylcholine (DPPC), supported bilayers can be formed at temperatures above the 41 °C gel–fluid transition temperature of this lipid, and subsequently brought to a lower temperature. This process produces a supported bilayer in the gel state. However, area changes of the bilayer during the phase transition can leave regions of the underlying substrate exposed (Tamm and McConnell, 1985). This is a problem for cell adhesion experiments since bare silica can strongly interact with cell surfaces. A useful consequence of the fluidity of supported bilayers is a self-healing tendency that tends to minimize defects, including scratches, in clean systems (Cremer et al., 1999).

The formation of planar bilayers by the vesicle fusion method maintains the orientation of the precursor vesicles. By tracking the orientation of transmembrane proteins that had been incorporated into vesicles with uniaxial orientation, it was observed that the outer surface of the vesicle becomes the top surface of the supported bilayer (Contino et al., 1994; Salafsky et al., 1996). Although these results concerning protein orientation are suggestive, little is known about the degree to which lipid orientation can be preserved when supported bilayers are formed from asymmetric bilayers. A variety of experiments using GPI-anchored proteins have demonstrated that these are highly mobile and accessible after supported bilayer formation. These proteins are generally incorporated into vesicles by mixing detergent solubilized protein with preformed small unilamellar vesicles or detergent solubilized phospholipids, followed by detergent dialysis (Mimms et al., 1981; Groves et al., 1996). Although the specific orientation of protein in the vesicles is not known, this process of lipid-linked protein incorporation and subsequent supported bilayer formation routinely produces supported bilayers with significant populations of protein available on the top surface. GPI-anchored proteins in silica-supported bilayers formed by liposome fusion are highly mobile with mobile fractions between 70% and 95%. This result suggests that the liposome fusion process strongly favors asymmetric distribution of the GPI-anchored proteins with a strong bias towards the upper leaflet of the bilayer. Proteins linked to phospholipids or glycolipids in the upper leaflet through noncovalent means are also laterally mobile. This includes antibodies bound to haptenated

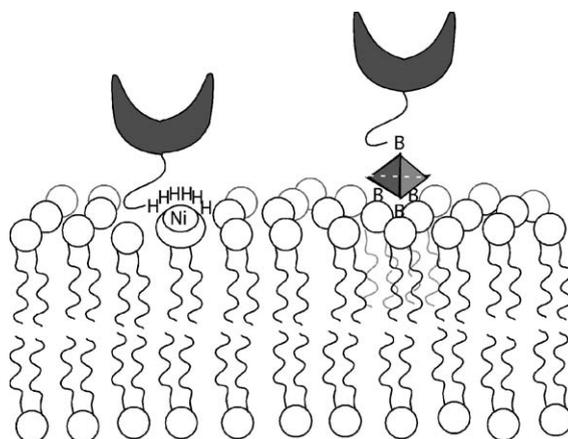


Fig. 2. General methods for attaching molecules to a planar bilayer. Two effective methods are based on interaction of His tag lipids with NTA and a second is based on an avidin bridge between biotinylated lipids and biotinylated proteins. A method for adding a single biotin to recombinant proteins is to add a BirA sequence that targets an enzymatic biotinylation system in bacteria. This method is widely used in the MHC tetramer technology that is used to detect antigen-specific T cells by flow cytometry. Biotins can also be attached to free cysteines. The ability to use the same reagents for flow cytometry and planar bilayer-based studies should broaden the appeal of the latter.

phospholipids (Humphries and McConnell, 1975), avidin bound to biotinylated lipids (Qin et al., 1995) and 6-histidine-tagged molecules linked to Ni^{2+} or Cu^{2+} chelating lipids (Celia et al., 1999). The latter two methods are of general interest for attachment of recombinant soluble proteins to the bilayer surface (Fig. 2).

8. Imaging supported bilayers

The planar geometry and proximity of the substrate greatly facilitate and enhance supported bilayer imaging capabilities. Leveraging this characteristic, a variety of techniques for acquisition of high-resolution and high information content images of supported bilayer systems are emerging. Conventional epifluorescence microscopy is widely utilized as a core methodology, which can also provide information about molecular diffusion from fluorescence photobleaching recovery (FPR) observations (Chan et al., 1991; Tamm and Kalb, 1993). When all the fluorescent molecules are confined to the bilayer, confocal

microscopy does not offer specific advantages, but can still be utilized. Scanning probe microscopy is becoming more widely applied to supported bilayer systems and complements optical imaging by extending resolution down to the nanometer length scale (Shao et al., 1996; Muresan and Lee, 2001; Yuan et al., 2002).

When molecules in cells or vesicles interacting with the bilayers need to be quantified, there are a number of choices to obtain the needed 3D information. Wide field epifluorescence illumination with deconvolution (Dustin et al., 1998), confocal imaging (Johnson et al., 2000), total internal reflection (TIR) illumination (Watts et al., 1986; Ajo-Franklin et al., 2001), reflection interference (Rädler and Sackmann, 1993; Albersdorfer et al., 1997; Grakoui et al., 1999; Kloboucek et al., 1999), fluorescence interference (Lambacher and Fromherz, 1996; Braun and Fromherz, 1998; Wong and Groves, 2001) and intermembrane fluorescence resonance energy transfer (FRET) (Niles et al., 1996; Wong and Groves, 2002) have all been applied. Each of these techniques offers distinct capabilities that can be matched to a broad range of experimental needs. For example, single molecule tracking can be performed in the TIR configuration. The short-range evanescent wave (~ 100 nm) near the interface substantially reduces background fluorescence, allowing single molecules in cell contact areas to be resolved (Klopfenstein et al., 2002). Epillumination and TIR can be used in combination to track cellular structures from areas outside the range of the evanescent wave that move into the interface, as has been observed with secretory vesicles (Schmoranzler et al., 2000; Toomre et al., 2000; Keller et al., 2001; Lampson et al., 2001).

Reflection and fluorescence interference microscopies can provide topographical information near a supported bilayer with nanometer precision. In the case of reflection interference, light waves reflected off of the inner surface of the substrate and the sample interfere. This can map the height of the cell surface above the interface and has been used to resolve topography of T cell synaptic junctions (Grakoui et al., 1999) and to study model systems consisting of a supported bilayer interacting with a giant vesicle (Kloboucek et al., 1999). Reflection interference relies on index of refraction differences between the cell membrane/cytoplasm and extracellular media to pro-

duce a reflected wave. No labels are required; however, multiple reflections can impair resolution in complex systems. For example, vesicles in the cytoplasm and the nucleus of cells frequently produce additional reflections. Fluorescence interference can achieve equivalent (or better) height resolution with the added benefit of specific fluorescent markers to isolate the structures of interest. This technique has been used to measure the gap between cultured neurons and silicon substrates (Braun and Fromherz, 1998) and to image spontaneously forming topographical structures involving two, interacting bilayers (Wong and Groves, 2001).

FRET has been applied extensively in biological studies to determine intermolecular separation distances with Angstrom precision (Stryer, 1978; Lakowicz, 1999). Recent studies of FRET between probes in two interacting lipid bilayers show promise for supported bilayer studies (Wong and Groves, 2002). A supported bilayer serves as the lower membrane in the junction. A second bilayer is then deposited by rupture of a giant vesicle. Both bilayers in the junction exhibit lateral fluidity. The upper bilayer is further able to undergo bending deformations, resulting in topographical structures, which can be resolved by intermembrane FRET. Molecules in the junction (e.g. glycolipids, proteins, etc.) induce topography based on their size. Size differences between molecules can be utilized to induce lateral organization (Wong and Groves, 2002). Labeling the molecules of interest is not required; fluorescent probes in the background lipid are sufficient.

9. Membrane domains in supported bilayers

In cell membranes, lipid raft domains are fundamental structuring motifs. Although not precisely defined, and likely representing several different types of structure, lipid rafts in cell membranes all appear to be based on lateral phase separation of cholesterol- and sphingolipid-rich domains from the more disordered phospholipid component of the membrane (Harder and Simons, 1997; Simmons and Ikonen, 1997; Kurzchalia and Parton, 1999; Radhakrishnan et al., 2000). These phase-separated domains have been implicated in a wide variety of cellular processes, including intracellular sorting of membrane

proteins (van Meer and Simons, 1988) and immune cell recognition (Montixi et al., 1998; Sheets et al., 1999; Viola et al., 1999). Careful study of the role of membrane lateral phase separation in cell signaling will be essential as these processes are unraveled.

Lipid phase separation can be observed in both giant vesicles and in supported bilayers (Dietrich et al., 2001; Feigenson and Buboltz, 2001; Rinia et al., 2001). Supported bilayers with well-defined phase segregation may be useful for the purpose of cell–bilayer interaction studies. However, the domain structures formed in synthetic mixtures of lipids appear to be much larger than those in biological membranes (Varma and Mayor, 1998). Physically, it is expected that the equilibrium size of phase-separated domains in bilayers will be large as the system minimizes energy associated with the phase boundaries. The existence of small equilibrium domain sizes (micrometers) in lipid monolayers is due to long-range electrostatic forces (McConnell, 1991); these forces may be greatly reduced in bilayers due to the presence of water on both sides of the bilayer. So far, experimental evidence in supported bilayers indicates that phase-separated domains grow to large sizes whenever mechanisms of equilibration are not arrested. Kinetically trapped configurations with small domains, however, are likely to be at least metastable. For example, mixtures of phospholipids, sphingolipids, cholesterol and phosphatidylinositol-3,4,5-trisphosphate form small domains that confine diffusion of pH domain bearing proteins (Klopfenstein et al., 2002). With more work, it may be possible to prepare model systems, out of equilibrium, with well-defined sizes of phase-separated domains.

10. Supported bilayers meet micro- and nanofabrication

An advantageous feature of supported bilayer systems is that structures on the supporting substrate can be used to impose order on the bilayer itself (Groves et al., 1997b, 1998b; Groves and Boxer, 2002). This relatively simple idea can be manifested in a wide range of configurations. At the most basic level, patterns of material that do not support bilayers (e.g. metals, proteins, etc.) are fabricated onto a surface, such as silica, that does support bilayers. The bilayer

blocking materials form barriers, which can be used to partition or corral the fluid bilayer in nearly any geometry. Deposition of a bilayer onto a grid of barriers produces an array of fluid, but isolated bilayer corrals (Groves et al., 1997b; Kung et al., 2000). Such micro- or nanopartitioned bilayers could prove useful for studies on protein pattern formation in cell–cell junctions.

Another aspect of patterned supported bilayers emerges when different bilayer compositions are deposited into corrals of an array. A number of techniques for printing such bilayer arrays have been developed ranging from sequential bilayer deposition (Dustin et al., 1997a), direct pipetting (Cremer and Yang, 1999; Groves et al., 2001) to microfluidic deposition (Kam and Boxer, 2000) and microcontact printing from a poly(dimethylsiloxane) stamp (Hovis and Boxer, 2000, 2001). A fundamental characteristic of these bilayer arrays is that multiple bilayer types are displayed, side-by-side, in a small area. The simplest version of this concept was used to test the effect of immobile MHC–peptide complexes on T cell migration (Dustin et al., 1997a). A ~ 1-mm diameter bilayer containing ICAM-1 and MHC–peptide complexes was formed in a parallel plate flow cells (bilayer 1). Once this bilayer was fully formed, the excess liposomes were washed out and then the flow cell was flooded with liposomes containing only ICAM-1. This resulted in the formation of a second bilayer (bilayer 2) adjacent to the first bilayer. A cell migrating across the boundary between bilayer 2 and bilayer 1 encounters a steep rise in the density of MHC–peptides complexes over a distance of < 1 μm . It was found that the T cells stopped migrating when moving from bilayer 2 to bilayer 1 (Dustin et al., 1997a). The sampling of the two domains is dependent upon T cell migration on ICAM-1, which averages ~ 10 $\mu\text{m}/\text{min}$. In this approach, bilayers 1 and 2 are large and continuous such that mobile molecules diffuse across the boundary forming a gradient spanning hundreds of micrometers. However, as expected, the transmembrane MHC molecules remained in place over many hours.

The ability to use micropatterned substrate and stamping technology allows for much more control of both diffusion and size of the domains. This allows each cell to sample and discriminate among different compositions before a response is triggered. For

bilayer patterns on the $\sim 100\text{-}\mu\text{m}$ length scale, Brownian motion, migration or tangential flow of the cells may allow multiple bilayer types to be sampled within seconds to minutes. Cellular discrimination in this context can yield results that differ from responses observed when a population of cells is first split into two groups, each of which is exposed to a single choice. For example, we have observed that HeLa cells adhere to and proliferate on supported bilayers that have been doped with phosphatidylserine (PS) (Groves et al., 2001). Without PS, supported bilayers generally block cell adhesion. The fidelity with which PS-free supported bilayers block cell adhesion depends on the proximity of preferable adhesion sites and other treatments of the bilayer. While cells do not readily adhere to bilayers in bovine serum albumin or casein containing media, the application of animal serum results in adsorption of vitronectin and fibronectin, which can mediate integrin-dependent cell adhesion (Bonte and Juliano, 1986; Smith et al., 1989; Wülfing et al., 1998).

Additional degrees of control over supported bilayer systems can be achieved by rearranging or altering the composition of bilayers after they have been formed. Electric fields applied tangentially to the supported bilayers can induce electrophoretic motion of lipids and proteins (Stelzle et al., 1992; Groves and Boxer, 1995). Applications of lateral electric fields include generation of continuous concentration gradients (Groves et al., 1996), molecular separations (van Oudenaarden and Boxer, 1999) or as an analytical tool to examine molecular mobilities and associations within the supported bilayers (Groves et al., 1997a, 1998a). Other areas of supported bilayer research include the generation of bilayers on electrically conductive substrates (Cornell et al., 1997; Wiegand et al., 2002). This enables application of an electric field perpendicular to the bilayer, which could facilitate analysis of ion channel proteins as well as providing an alternative way of perturbing selected regions of a supported bilayer and nearby cells.

11. How bilayer patterning will interface with immunobiology

Basic research in the near future will determine the ability of planar bilayers and micropatterned sub-

strates to control biological responses *in vitro*. The first wave of questions will be very basic in nature and will involve the application of micropatterning to hypothesis testing. For example, the formation of the immunological synapse involves movement of MHC–peptide complexes from the periphery to the center of the synapse, a distance of about $5\ \mu\text{m}$ (Grakoui et al., 1999; Krummel et al., 2000). The importance of this movement is not known and has been difficult to test. Patterned substrates offer an opportunity to directly test the basis and importance of this movement process (Fig. 3). Beyond this basic research, there are also a number of medical applications that should be explored as our knowledge of these systems increases. Goals related to the immune system will be the fabrication of substrates that induced different classes or modes of T cell responses in conjunction with specific cytokine treatments. Important goals would be the induction of antigen-induced cell death, T cell anergy, T helper 1 responses (interferon- γ), T helper 2 responses (interleukin-4), effective antitumor responses and regulatory T cells that suppress responses in an antigen-specific manner. These technologies could be applied in both diagnosis and therapy since appropriate substrates would be able to readout the immune status based on assaying T cell function in blood or might be used to produce large numbers of cells *in vitro* for transfer back into patients to boost or regulate immune responses. These technologies could then be introduced into *in vivo* systems

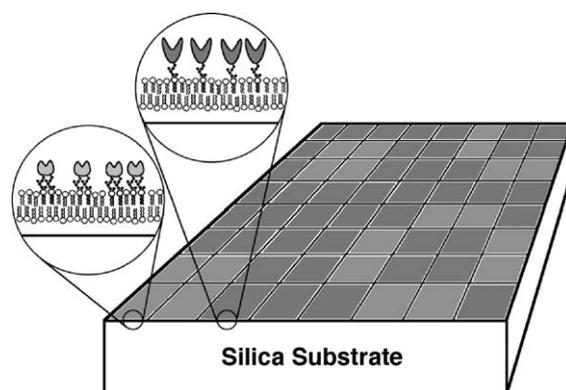


Fig. 3. Patterned substrates to test the role of protein movement in the immunological synapse. Prepatterned substrates can be generated with ICAM-1 (dark gray) and MHC–peptide complexes (light gray) in nascent or mature synapse patterns to test the role of the ordered formation process.

utilizing recently described *in vivo* imaging to directly assess how micropatterned systems interact with immune cells in the more complex *in vivo* environment. Since many effector cells are in tissues, it may be possible to track effector responses with small micropatterned probes that would require only a pinprick for application. Micropatterned systems on small beads might act as long-lasting immune stimulators for *in vivo* application. The next 10 years in this field should be an exciting time as these and other imaginative applications are tested.

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