Diffusion of Supported Lipid Bilayers on pH Responsive Polymer Cushions Characterized by Fluorescence Recovery After Photobleaching

By

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ABSTRACT

Biological membranes are extremely complex, highly organized, responsive thin films composed of a myriad of lipids and proteins. Due to their complexity, supported lipid bilayers have been used as alternates to living cell membranes for study of membrane dynamics, membrane structure, and lipid-protein interactions, among other topics. The tools of modern genetic engineering and bioorganic chemistry allow us to couple many types of biomolecule to supported membranes. This results in wide variety of interfaces that can be used to control, organize and study the properties and function of membranes and membrane-associated proteins. The overarching goal has been to develop robust and easily fabricated biomimetic membrane platforms to facilitate the study of transmembrane proteins for sensing applications[1-2].

Despite the large volume of research, development of solid supported membrane bilayers that recreate the proper environment has proven to be difficult[3]. Some fundamental drawbacks have been protein substrate interactions leading to premature denaturing of the protein and minimal-to-no mobility within the membrane. To ameliorate these issues, polymer supported lipid bilayers are widely used. One of the main attractions for using polymeric cushions is to mimic thermodynamic and structural properties of free bilayer more specifically the cytoskeleton and extra cellular matrix, which is an important feature of the native cellular environment[4].

Chapter 1 describes the fabrication and characterization of polymer supported bilayers and reviews the lateral mobility of the lipids and proteins on various types of

polymer supports. Also presented are potential practical applications including biosensors and future directions of development of polymer supported lipid bilayers. Chapter 2 provides the mobility of a polymer supported lipid bilayer with a polymer cushion that is pH responsive and structurally tunable in order to provide an environmentally sensitive interface. In details, the simple preparation of poly(acrylic acid) (PAA) cushioned membranes is described. The PAA cushion provides a tunable[5], water rich environment for supported lipid membrane applications. Conveniently the thickness of the PAA cushion can be controlled by manipulating the pH of the surrounding aqueous solution and the concentration of the spin-coating solution. As demonstrated in this work, the structure and continuity of cushioned membranes remains intact and this approach offers a robust and facile means to fabricate biological membranes for biophysical studies and sensing applications.

CHAPTER 1: LITERATURE REVIEWS ON POLYMER SUPPORTED LIPID BILAYERS

1.1 Abstract

The lipid bilayer is a universal basis for cell-membrane structure. Its role is critical because its structural components provide the barrier that marks the boundaries of cells and sub-cellular compartments, regulates the movement of materials into and out of cells, as well as cellular communication. Supported lipid bilayers provide an excellent model system for studying the physics, thermodynamics, and surface chemistry of cellular membranes. Moreover, the control and planarity of the support enables the utilization of a wide variety of surface-specific analytical techniques. This makes it possible to study fundamental properties of membranes and their constituent lipid and protein molecules and to investigate processes such as cell signaling and ligand-receptor interactions.

A recent focus in supported lipid bilayer-based model systems is the incorporation of transmembrane proteins in their native form. Towards this end, various types of polymer layers are being explored to cushion the membrane and prevent substrate-protein interactions, which have been shown to lead to protein denaturation and immobility. Specifically, a review of the lateral mobility of the lipids and proteins on polymer supported bilayers is provided in Chapter 1 as a basis for the novel polymer cushioned membrane work described in Chapter 2. In addition, a summary of potential applications and future directions in this field is given.

1.2 Introduction

Model bilayer systems allow for the simplified investigation of biological processes that occur at the cellular level. In the 1980s, Tamm and McConnell[6] developed the first solid supported membrane by directly depositing a lipid bilayer onto a glass support. Since their inception solid supported lipid bilayers have been widely used as model systems for cellular membranes. This opened the door for studying lateral organization and dynamics of lipids and proteins in membranes. Although this strategy has been successfully used to transduce cell membrane potentials to electric circuits[7] or to study questions of cell-cell interaction from the immune system[8], research in this field is still in the early stage. This is primarily because current model systems are far too simple to mimic the complex interplay of the broad variety of complex molecules in native biological membranes.

The simplest approach to mimicking a biomembrane on a solid substrate is with a solid-supported, single phospholipid bilayer. If the substrate is sufficiently hydrophilic, an ultrathin water layer of <10 Å is present that acts as a lubricant between the bilayer and the substrate[9-10]. Still, strong frictional coupling between the bilayer and the underlying substrate occurs, impeding the lateral diffusion by several orders of magnitude, which is accompanied by a break-down of the 2D fluid nature of the membrane[11-12]. Furthermore, it has been observed that proteins with large membrane anchors and integral membrane proteins are immobile in supported bilayers presumably a result of interactions between the membrane protein and the solid support, which may reduce or eliminate the protein function[13].

One way to overcome these limitations is to increase the thickness of the aqueous lubricant layer by introducing a hydrophilic polymer cushion between the membrane and the solid support. The polymer film, which acts as a spacer, minimizes negative substrate effects, such as defect formation, decreased lateral mobility, and limited self-healing of the membrane, resulting in better biomembrane properties. Ideally, when transmembrane proteins are embedded into a polymer supported lipid matrix, they are expected to show long-range lateral mobility under non-denaturing conditions. To this end, Ringsdorf's group laid the foundation for polymer supported phospholipid bilayers on planar solid substrates[14-15]. Two styles of polymer cushions are typically used. In the first, a polymer layer is deposited on the substrate and the membrane is subsequently fabricated on top of the cushion. In the second, polymer functionalized lipids are deposited or incorporated into a membrane, that act as tethers, which suspend the membrane over the support. Regardless of the approach, one of the most desired characteristic features of supported membrane is to maintain membrane fluidity and lateral mobility of any embedded proteins. Therefore, the focus here is on the studies of lipid and protein mobility in polymer supported membranes. For comparison, some background on membranes directly supported on solid supports is first provided.

1.3 Solid Supported Lipid Bilayers

Solid supported lipid bilayers prepared by depositing phospholipid bilayers onto various solid substrates represent an important class of model systems in membrane

research. Not only have they provided insights to cellular process such as cell signaling[8] and adhesion[16] but the solid support also increases the robustness and stability of the membrane. The supported phospholipid membrane interacts with the underlying solid support through a combination of van der Waals, electrostatic, hydration and steric forces[17]. It is believed that the fluidity of the membrane is maintained by a ~10 Å layer of trapped water between the substrate and the bilayer[18]. In order to maintain the high lipid mobility the surface of the support should be hydrophilic, smooth, and clean. Common substrates that have been used are borosilicate glass[17,19], mica[20-21], quartz[6,22], and oxidized silicon[6]. Other studies have used thin films on solid supports as observed with TiO_2[23-24], indium-tin-oxide[25-26], gold[27], silver[28], and platinum[29]. Supported lipid bilayers also have been successfully formed on silica, polystyrene, and magnetic beads whose sizes range from tens of nanometers to several micrometers[30].

Three common methods exist for the formation of phospholipid bilayers on planar supports. They are (1) Langmuir-Blodgett (LB) and Langmuir-Schaefer (LS) transfer of two consecutive lipid monolayers to the substrate to form a bilayer (2) a spontaneous spreading of unilamellar vesicles on the substrate surface and (3) a combination of Langmuir-Blodgett monolayer transfer and vesicle fusion can be used to form supported phospholipid bilayers. The first method involves the transfer of individual monolayer leaflets of lipids from the air-water interface by the Langmuir-Blodgett technique. This is carried out by pulling a hydrophilic substrate through a lipid monolayer. Langmuir-Schaefer technique is the horizontal dipping of the substrate to create the upper leaflet. Historically Tamm and McConnell[6] were the first to apply the Langmuir-Blodgett

deposition method to deposit a lipid bilayer onto quartz, glass and oxidized silicon substrates. Although Langmuir-Blodgett and Langmuir-Schaefer methods are useful for the deposition of lipid bilayers and can be used to fabricate asymmetric bilayers[31], it is difficult to incorporate transmembrane proteins with this technique because portions of the transmembrane proteins within the membrane are exposed to air and can become irreversibly denatured[13].

The second method of supported bilayer formation is the adsorption and fusion of vesicles from an aqueous suspension to the substrate surface. This method was first used by Brian and McConnell to study immune response and is now a widely used standard technique[10]. It is one of the easiest and most versatile means for forming solid supported lipid bilayers. Vesicles are typically prepared by extrusion of multilaminar vesicles through porous polycarbonate membranes at high pressure[32], sonication of aqueous lipid suspensions[33], freeze-thawing small unilamellar vesicles (SUV) into large unilamellar vesicles (LUV)[34], or a combination of these. Membrane formation and spreading on hydrophilic substrate takes place by simply exposing the substrate to the vesicle suspension. Even though it is simple, this method tends to result in bilayers with more defects because there are so many factors affecting the adsorption and fusion of vesicles to solid supports[24,35] (e.g. vesicle composition, size, surface charge, surface roughness, solution pH, etc.). A significant advantage, however, is that integral membrane proteins can be integrated into the vesicles and deposited with the lipids on the substrate. Although the orientation of membrane proteins cannot be controlled through this method[36], asymmetry in the protein shape and extracellular structure frequently results in

a higher propensity for one orientation versus another.

A combination of Langmuir-Blodgett to form the inner leaflet and fusion of vesicles to the pre-deposited monolayer is also a highly efficient method for the formation of asymmetric bilayers[31] and for the incorporation of transmembrane proteins into solid supported bilayers[22]. Since, the vesicles to be fused will encounter the hydrophobic fatty acyl chains of the supported amphiphilic monolayer rather than the hydrophilic substrate, the proteins tend to be unidirectionally oriented in the supported bilayer[37-38].

One important characteristic of supported membranes is the lateral mobility of their constituent molecules. Fluorescence recovery after photobleaching (FRAP) and single particle tracking (SPT) are two commonly used experimental methods to measure the lateral diffusion coefficients of fluorescently labeled lipids or proteins in supported bilayers. In FRAP, an intense laser light is used to flash partially in a small area of the sample containing fluorescently labeled molecules such as NBD, Texas Red and Rhodamine; thereby "bleaching" the fluorescently labeled molecules in that region. After bleaching, a low intensity laser light is used to follow the recovery of fluorescence caused by diffusion of molecules outward of the bleached region and the inward diffusion of neighboring non-bleached molecules[39]. The diffusion coefficient and mobile fraction are extracted from the time course and the amplitude, respectively, of the recorded recovery curve[40]. Unlike FRAP, where it follows the dynamics of an ensemble of macromolecules, SPT follows the dynamics of individual particle in the cell membrane tagged with latex beads, colloidal gold, or fluorophores, whose trajectories are monitored by computer assisted video microscopy. SPT is the preferred technique when detail information on

different populations of moving particles in a heterogeneous system is required. Although the information content of SPT is much higher than that of FRAP, it is much more demanding on instrumentation and statistical evaluation procedures. It has been illustrated that SPT can yield erroneous diffusion coefficients when inappropriate sampling frequencies are used, and that noise can lead to errors in accurately finding the actual particle position, which consequently leads to apparent subdiffusion[41-42]. Other methods to measure the lateral diffusion of the lipid bilayers includes using pulse saturation recovery electron spin resonance[43], pulsed field gradient magic-angle spinning nuclear magnetic resonance (PFG-MAS NMR)[44], fluorescence correlation spectroscopy (FCS)[45] and more.

A wide variety of additional analytical tools are commonly used methods to characterize the structure of supported membranes. The high normal resolution of atomic force microscopy (AFM) has been used to image domain structure in supported lipid monolayers and bilayers in fluid environments on the basis of topographic height differences between gel phase, liquid-ordered, and liquid-disordered domains[46]. Neutron reflectivity has been used to characterize the transverse organization of supported bilayers structurally. The intensity of neutrons reflected from a surface yields information on the scattering-length density profile normal to the surface, from which the structure at the interface as well as roughnesses of the interfaces can be deduced[47]. Fluorescence interference-contrast (FLIC) microscopy is another means to measure the vertical displacement of the bilayer from the surface of an oxidized silicon chip by the interference pattern between the incoming and reflected light[38]. In some cases, it is

desirable to discriminate surface from bulk fluorescence to visualize cellular trafficking occurring at the model membrane or cell surface without interference. Total internal reflection fluorescence (TIRF) microscopy can conveniently achieved this by selectively exciting fluorophores near the solid surface (within 100nm) without exciting fluorescence from regions farther from the surface[48]. Nuclear magnetic resonance (NMR) has been used as powerful tools for investigating the structure and the dynamics of single lipid bilayers on a spherical solid support and determining structures of proteins and protein complexes that are readily soluble in aqueous solution[49]. Absorption bands of liquid water overlap with several bands that are of interest in protein and membrane spectroscopy. To minimize these problems, FTIR spectroscopy allows for accurate background subtraction and the reliable recording of protein spectra in aqueous solutions[50].

Solid supported lipid bilayers allow facile control of membrane composition, increase in the robustness and stability of the supported phospholipid bilayer membrane, and enable the properties and function of the membrane and its constituent molecules with a host of surface sensitive techniques. Another advantage of using solid supported lipid bilayers is to be able to probe how membrane structure is altered by changes in environmental conditions and/or through the binding of different molecules. However, the major disadvantage is that the supported membrane is not truly decoupled from the underlying substrate. Because of this, transmembrane proteins embedded in the membrane may interact unfavorably with the underlying substrate and cause proteins to become immobile and hinder their function.

1.4 Polymer Supported Lipid Bilayers

While solid supported phospholipid bilayers are excellent platforms for investigating many cellular processes, they have difficulty mimicking the appropriate environment for transmembrane proteins, especially those protruding from the membrane. The ~10 Å water layer that resides between a phospholipid bilayer and a solid support provides lubrication and maintains sufficient mobility for the lipid molecules[9-10]. However, the underlying water layer is not sufficient to prevent many peripheral portions of transmembrane proteins from immobilization or denaturation if they come in contact with the substrate[13]. The desire to harness the beneficial properties of supported membranes for integral protein studies has been the driving force for the development of the polymer supported bilayer systems.

In cells, the cellular membrane is supported by an underlying three-dimensional protein network called the cytoskeleton, which provides the distinct shape to the cell and supports the two-dimensional lipid bilayer. To mimic the physical properties of a cytoskeleton, a well designed polymer cushion should act as a deformable and mobile substrate. In general, a successful polymer support for transmembrane proteins is hydrophilic, flexible, not highly charged, not strongly cross-linked and does not interact strongly with membrane lipids or proteins[1]. Even weak interactions between the phospholipid bilayer and the polymer support can result in an unstable system. However, for robustness in practical applications, the polymer layer can be covalently attached to the solid surface and/or be composed of polymer anchored lipids or alkyl side chains capable of inserting into the phospholipid bilayer. There are several types of polymer

cushions that have been explored for supporting phospholipid bilayers. These include polysaccharides dextran[1,51], chitosan[52], agarose[53], cellulose[54], polyelectrolytes[14,55-60,64], and lipopolymer tethers[15,65-71]. Over the past decade, extensive researches have been done on polymer supported membrane using polyelectrolytes and lipopolymers (lipids bearing polymer groups) as common polymer cushion materials.

Polyelectrolyte cushions can be directly adsorbed from solution to a variety of substrates by means of electrostatic layer-by-layer deposition, providing remarkable control over the film thickness and polymer composition[2]. In turn, van der Waals, hydrogen bonding, as well as electrostatic interactions adhere the lipid layer to the polymer. When the polyelectrolyte layer is deposited onto a substrate, charge on the surface builds up and repels other materials with the same charge away from the interface. Since attractive electrostatic interactions govern the formation of the polyelectrolyte multilayers, the thickness of the multilayer assembly depends mainly on the surface and polyelectrolyte charge density, which can be controlled by the pH and ionic strength of the deposition medium. On the other hand, if excessive charge builds up it can adversely affect the function and mobility of membrane constituents and alter interactions between proteins and the supporting cushion[2].

Ringsdorf and coworkers were one of the first to work on polymer supported membranes on a solid substrate[14,55]. Polyethyleneimine (PEI) was initially used as an interlayer between a solid support and an amphiphile membrane. Majewski et al. confirmed that the zwitterionic lipid dimyristoylphosphatidylcholine (DMPC) formed inhomogeneous multilayer structures when deposited on a PEI covered solid substrate[56-57].

Subsequently, a weak polyelectrolyte, poly(methacrylic acid) (PMA) was demonstrated to provide a cushion capable of supporting a DMPC bilayer on silicon wafer[58]. However, despite numerous theoretical and experimental studies devoted to this subject little lateral mobility information has been reported concerning the influence of the polyelectrolyte cushion on the mobility of the lipid bilayers. In one case, Cassier and co-workers[59] fabricated lipid bilayers with 10% charged dioleoylphosphatidic acid (DOPA) and zwitterionic lipid DMPC on the polyelectrolyte multilayers (PEM). The polyelectrolyte support was prepared by layer-by-layer deposition of positively charged poly(allylamine hydrochloride) (PAH) and negatively charged poly(styrene sulfonate sodium salt) (PSS) onto the PEI-coated glass or cysteamine(Cys)-coated gold substrates. They reported the diffusion coefficients to be above 1 $\mu m^2/s$ for temperatures above the phase transition temperature. A similar approach used DOPA, DMPC and dioleolylphosphatidylcholine (DOPC) on PAH/PSS multilayers supported on PEI-coated glass or silicon substrates[60]. Measured mobility of the lipids was on the order of 10^{-3} $\mu m^2/s$, which is much less than typical values of lipid molecules in monolayers or bilayers for fluid phase or gel phase. Diffusion coefficients of fluid phase DMPC and DOPC bilayers supported on bare silicon wafers were on the order of 1–8 $\mu m^2/s$[6]. Other reported values for bilayers of DMPC on silicon wafers and palmitoyloleoylphosphatidylcholine (POPC) on quartz were 3–4 $\mu m^2/s$[22,61] and bilayers of POPC and DPPC on silica particles showed similar values[62-63]. Another choice of forming PEM was to adsorb alternating layers of cationic poly(diallyldimethylammonium chloride) (PDDA) and anionic PSS onto mercaptoundecanoic acid coated gold[64]. Anionic lipid Stearoyloleoylphosphatidylserine

(SOPS) and zwitterionic lipid POPC were mobile at room temperature with lateral diffusivities of approximately 0.1–0.2 $\mu m^2/s$.

Lipopolymers are another popular class of polymer cushion. They consist of lipids with macromolecular groups coupled to the head group which can be inserted into a phospholipid membrane and covalently tether both the polymer-substrate and polymer-bilayer interfaces. Unlike polyelectrolyte cushions, 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[65]. Attachment of a lipopolymer to the solid substrate has been carried out through sulfur–metal bond formation[15,67-69], silane bonding[65- 66 ,70-71], or photocross-linking[65,71]. Some common polymer backbones used in the synthesis of lipopolymers are acrylamide[15,66-68], poly(ethylene glycol) (PEG)[69-70], and ethyloxazoline[65,71].

In other pioneering work by Ringsdorf's group[15] they synthesized a multifunctional hydrophilic polymer containing disulfide anchor groups and hydrophobic alkyl chains that could self-assemble on gold substrates. The alkyl chains would insert into the membrane, stabilizing a cushioned membrane structure on the gold surface. Adopting this method several other authors have attempted to use self-assembly of disulfide-containing lipopolymers to gold surfaces as well[67-69]. Seitz and coworkers[68] formed DMPC bilayer onto a lipopolymer containing monolayer, which was comprised of an acrylamide backbone modified with lipid side chains of dimyristoylphosphatidylethanolamine (DMPE) and a disulfide moiety for the chemisorption to gold or mica substrates.

Additionally, chemisorbed layers of PEG were formed on gold films where the PEG films were prepared by co-adsorbing a telechelic PEG lipopolymer DSPE-PEG-PDP and a non-lipid functionalized PEG-PDP from an ethanol/water mixture. Diffusion of lipid bilayers were found to increase to ~2 $\mu m^2/s$ compared to 0.5 $\mu m^2/s$ on a bare glass surface[69]. A similar approach by Wagner and Tamm[70] utilized a telechelic PEG chain functionalized with a lipid on one end and a trimethoxysilane on the other (DMPE-PEG-triethoxysilane, DPS). Uniform POPC bilayers with lateral diffusion coefficients of 0.8–1.2 $\mu m^2/s$ were observed when the DPS concentration was kept slightly below the mushroom-to-brush transition. Shen et al.[71] and Naumann et al.[65] used a benzophenone-silane molecule to photochemically attach both random copolymers and end-functionalized polymers containing lipid-like alkyl moieties to surfaces. Shen et al. found the mobility of the lipid bilayers to be in a order of ~0.1 $\mu m^2/s$, whereas, Naumann et al. found the values to vary in the range of 1–17.7 $\mu m^2/s$ depending on the tethering densities (lipopolymer/ phospholipid molar ratios). Overall, a number of chemistries have proven to yield reasonable diffusion coefficients of lipopolymer containing bilayers.

In summary, various polymer layers have been used as hydrated spacers to lift membranes from solid surfaces. The hydrated space created by the addition of a polymer layer or tether is not only advantageous for decreasing the substrate effect on the membrane itself but also still allows analytical techniques for investigation of biological processes in membranes. Moreover, such systems are a closer mimic of the polymer like cytoskeleton structure that supports the cell membrane and maintains the activities of incorporated biomolecules. For example, transmembrane proteins that protrude from the

membrane can avoid denaturation and immobilization that frequently occurs when there is direct interaction with the solid substrate.

1.5 Transmembrane Proteins Mobility on Polymer Supported Lipid Bilayers

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 into the bilayer, especially those with large extracellular domains, it may be necessary to use a polymer supported bilayer to prevent protein denaturation on the underlying substrate.

Despite extensive work on cushion and tethering designs, only a few studies describe the use of these systems for studying the lateral mobility of transmembrane proteins. Wagner and Tamm[70] reported the diffusion coefficients of cytochrome b_5 and annexin V on PEG-conjugated phospholipid membrane. They found 25% of the cytochrome b_5 molecules diffused at a rate of ~1 $\mu m^2/s$, which is in the same order of magnitude as the phospholipids diffusion[6,22,61-63]. The rest of the cytochrome b_5 molecules diffused several orders of magnitude more slowly or not at all, presumably as a result of interactions between the proteins and the underlying polymer or substrate. Likewise, annexin V diffused in a range of 10^{-2}–10^{-1} $\mu m^2/s$. A similar approach by Diaz and coworkers[72] measured the mobility of annexin V on double cushioned bilayer, which was formed by first adsorbing a polymer layer of bovine serum albumin (BSA) onto the solid substrate and then fusing PEG-conjugated lipids on top of the first layer. A diffusion coefficient of ~3 $\mu m^2/s$ for annexin V was obtained, which resembles the diffusion of lipids. In addition,

the PEG tethered system was used for the reconstitution of mobile SNARE proteins[37]. About 75% of the reconstituted proteins were laterally mobile with a diffusion coefficient of ~0.75 $\mu m^2/s$ in a phosphatidylcholine lipid bilayer. Another protein mobility study investigated human blood platelet integrin receptors $\alpha_{IIb}\beta_3$ in a supported bilayer system that rested on a cellulose cushion[73]. Again, only 25% of the integrin receptors were mobile, exhibiting diffusion coefficient of 0.6 $\mu m^2/s$. Instead of using a planar substrate, Davis and coworkers[74] investigated retinal protein, bacteriorhodopsin (bR) containing unilamellar phosphocholine lipid bilayers on nanoporous silica microspheres. The measured diffusion coefficient was 0.038 $\mu m^2/s$, which is about two orders of magnitude smaller than diffusion of unilamellar phosphocholine lipids on planar solid supports. In this case, however, no polymer layer was used to cushion the membrane on the nanoporous silica microspheres. In general, the mobility of various transmembrane proteins has been shown to be suppressed or largely immobilized in polymer cushioned membrane systems. An imbalance in the stabilization forces or a large number of tethering molecules can decrease the mobility of the supported lipid bilayer and alter the phase transition temperature. Even in some cases polymer supported lipid bilayers are less stable than those formed directly on a solid substrate and often possess more defects[1]. In addition, structural characterization is frequently insufficient to quantitatively establish that the membrane is uniformly cushioned or that portions of the membrane are not in contact with the underlying substrate.

Although supported membranes have been used in fundamental and applied studies of membrane structure and dynamics, lipid-protein interactions, ligand-receptor interactions,

and development of membrane-based biosensors, a well-defined cushioned membrane system for the presentation of transmembrane proteins with large peripheral domains has yet to be established.

1.6 Applications and Future studies

A supported membrane was first used to investigate cellular immune responses and has been used extensively to study molecular interactions at interfaces as a model for cell–cell interaction[75]. Examples include Fc receptor-mediated adhesion and signaling and formation of the immunological synapse between T cells and antigen-presenting cells[76]. The advantage of a polymer supported bilayer system is the ability to control the bilayer composition and the surface that responses to subtle variations in external stimuli such as temperature, pH and electric field. One example of a device application is a biosensor that uses ion-channel switches[77]. The active elements of the ion-channel switch comprise a gold electrode to which is tethered a lipid membrane containing gramicidin ion channels linked to antibodies. The conductance at the electrode is governed by the ion channels in the membrane which can be switched by the recognition event. The sensor is essentially an impedance element whose dimensions can readily be reduced to become an integral component of a microelectronic circuit. It may be used in wide range of applications ranging from cell typing, to detection of a wide array of biological molecules including proteins, viruses, antibodies, DNA, electrolytes, drugs, pesticides and other low-molecular-weight compounds.

To date, most studies have focused on scientific applications, for example, the design

of cell surface models to study fundamental aspects of cell adhesion. At present, more work is required to improve the stability of soft interfaces under physiological conditions and to develop methods for self-restoration of damaged films before technical applications under real conditions become widely feasible. However, one can anticipate more growth and an even more prominent role of polymer supported bilayers in basic and applied membrane research in the decades to come.

CHAPTER 2: LATERAL DIFFUSION OF POLYMER SUPPORTED LIPID BILAYERS

2.1 Abstract

The lateral mobility in a model membrane consisting of phospholipid bilayers attached to a polyacrylic acid (PAA) cushion was studied by means of fluorescence recovery after photobleaching (FRAP). Lateral lipid diffusion coefficients provide a quantitative measurement of the interfacial interaction of lipid layer and polymer cushion. In this chapter, the effect of PAA thickness and solution pH on the mobility of the membrane is determined. Diffusion measurements are further performed in aqueous solutions of neutral pH and higher salt concentrations to investigate their influence on diffusion of supported lipid bilayers at physiological conditions.

The environmental pH and resulting alterations of the PAA cushion was found to significantly influence membrane diffusion. For example, at low pH, when PAA is collapsed, the diffusivity was found to be significantly slower than when under conditions of high pH when the PAA deprotonates and swells. Simply increasing the thickness of the PAA cushion by increasing the amount of PAA on the surface was found to have negligible effect on the lipid bilayer diffusion. Under physiological conditions, lipid diffusion was found to be essentially indistinguishable between membranes on a PAA support or on a glass substrate. Together, these results show that PAA with a pH responsive, structurally tunable interface has potential applications for membrane based bio-chemical sensors and as a platform for biophysical studies of membranes.

2.2 Introduction

Cellular membranes are vital components of all living systems. They consist largely of a phospholipid bilayer with embedded proteins[4]. Lipids provide mechanical stability and specific proteins aid in signal transduction which allows the cell to control membrane transport and regulate many important biological processes at membrane surfaces[78]. A large research effort has been focused on understanding lipid membranes yielding insight into lipid composition of membranes[79], lipid transmembrane asymmetry[80], and their heterogeneous lateral distribution[81]. However, several fundamental questions remain unresolved due to the complexity of cellular membranes and their interactions with intra- and extracellular networks. For example, information on the membrane dynamics or structure in a native system is limited. In addition, lipid-protein interactions at the molecular level, and lipid-lipid interactions in complex mixtures are not well understood.

To address these, and other fundamental questions about membrane systems, researchers have frequently simplified the system to its most basic component, a single lipid bilayer. Moreover, solid supported lipid bilayers prepared by depositing phospholipid bilayers onto various solid substrates including glass[10], mica[21], and fused quartz[22], have been most widely used as models for cellular membranes. For almost 25 years, solid supported lipid bilayers were used successfully to study the cellular membrane because of their advantage of increasing the robustness and stability of the membrane and facilitating the utilization of analytical tools such as atomic force microscopy (AFM)[46], neutron reflectivity[47], nuclear magnetic resonance (NMR)[49], fourier-transform infrared spectroscopy (FTIR)[50], Neutron , etc,.

One approach to characterize the interaction of lipids with other components in the membrane and the support is to determine the lateral diffusion coefficient of lipids in the bilayer. Lateral lipid diffusion is of particular interest because it plays an important role in cellular process such as cell signaling[8] and adhesion[16]. Fluorescence recovery after photobleaching (FRAP) is commonly used technique to study the mobility of fluorescently labeled molecules[39-40]. Lipids can be labeled with a fluorochrome dye which will fluoresce when irradiated by light. In a FRAP experiment, fluorescent lipids are irreversibly photo-bleached in a small area of the membrane by a high-powered focused laser beam. Subsequently, fluorescence recovers due to lateral diffusion of surrounding non-bleached fluorescent lipids into the bleached area, which is recorded at low light intensity.

Although FRAP experiments have a long history on solid supported lipid bilayers they have one fundamental drawback. The ~10 Å thin film of water that separates the phospholipid bilayer and the solid support provides lubrication and supports lateral mobility of lipids in the bilayer. However, it is insufficient to screen molecules or proteins that protrude from the membrane[9-10]. As a result, substrate-exposed portions of transmembrane proteins incorporated in the bilayer interact with and are frequently pinned to the hydrophilic substrate. Deleterious protein–substrate interactions commonly result in denaturation and immobilization of proteins in contact with the substrate[13].

To alleviate these problems, several attempts have been made in recent years to develop an easily fabricated biomimetic membrane platform that maintains proper protein structure, function and mobility. The general strategy for increasing the spacing between

the solid substrate and membrane is to create a water rich polymer spacer or tether region to cushion the membrane[1].

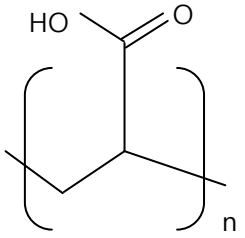

Figure 1. Structure of Polyacrylic acid (PAA)

In this work, a polymer cushion based on polyacrylic acid (PAA) was selected because of a few unique properties, which are especially advantageous for supported membrane studies. PAA is readily available in a wide array of molecular weights, and thus does not require custom synthesis. The degree of ionization of the carboxylic acid groups in the PAA can be tuned by varying the pH of the aqueous solution. As the pH increases, PAA chains stretch due to the ionization of most acid groups. Therefore, slight change on the pH of the aqueous solution can significantly affect the thickness of the PAA cushion[82-83]. This pH dependence of the polymer cushion thickness can aid in many membrane studies. For example, it will be able to provide a sensitive and convenient method to measure hydrogen-ion transport across the membrane. Another means to modify the thickness of the PAA cushion is to vary the concentration of PAA in the spin coating solution. The thickness of the film was measured by neutron reflectivity by other members of the Kuhl group which shown a linear dependency to the concentration of the solution. This is in a good agreement with other studies[84-85]. Thus, the thickness of the PAA cushion can be conveniently controlled simply by manipulating the concentration of the spin-coating solution. This provides a straightforward means to tailor the thickness of the cushion for different applications. In addition, simple amide coupling reactions with the available carboxylic acid groups provide a convenient means to functionalize the PAA film with

molecules of interest. PAA is easily deposited as a uniform thin-film from solution casting. Because of these properties, PAA can be fabricated to present a structurally tunable and environmentally sensitive interface for supporting biological membranes.

In this chapter, results of an investigation to quantify the interactions between the membrane bilayer and the underlying polymer cushion, in the absence of proteins, is reported. The pH dependence of the mobility of the lipid bilayer on PAA cushions is determined in detail. Based on FRAP studies, it is demonstrated that the lateral mobility of the supported lipid membrane can be tailored via pH from (1) a relatively fast diffusion at physiological conditions equivalent to diffusion rates obtained on glass in the absence of a cushion to (2) a relatively slow diffusion rate under acidic conditions when the PAA cushion is collapsed. No dependence of membrane diffusion on the deposited PAA film thickness was observed demonstrating that the film thickness can be tailored a prior for the desired application.

2.3 Materials

1,2-dimyristoleoyl-sn-glycero-3-phosphocholine (DMPC) and Texas Red® DPHE 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine were purchased from Avanti Pol ar lipids (Alabaster, AL) and Invitrogen (Carlsbad, CA). Lipids were dissolved in Chloro form, HPLC grade, purchased from Fisher Scientific (Fairlawn, NJ) to make solutions at a final concentration of 1 mg/ml. Poly(acrylic acid) (PAA 450k MW, 0.1% cross-linked) a nd aminopropyltriethoxysilane (APTES) were purchased from Aldrich (St. Louis, MO) a nd used without further purification. 1 inch diameter circular fused quartzes (thickness of 0.5 mm) and 18 mm diameter circular microscope cover glass (thickness of 0.2mm) were purchased from Mark optics (Santa Ana, CA) and Fisher Scientific (Pittsburgh, PA).

2.4 Methods

2.4.1. Preparation of PAA Coated Surfaces

Prior to poly(acrylic acid) (PAA) deposition, all the glass substrates were cleaned according to the following procedures to remove any organics. First, the substrates were s onicated for 10 min in a strongly basic detergent (Hellmanex) diluted with excess amount s of Nanopure water. The surfaces were extensively rinsed with Nanopure water followed by an additional 10 min sonication in acetone. After a final sonication in isopropanol, the substrates were rinsed with Nanopure water and dried with Nitrogen (N2). Finally, the su bstrates were cleaned in a UV-Ozone chamber (Jelight Company Inc., Irvine, CA) for 20–30 min.

The UV treated substrates were then silanized with solution of 0.1% APTES in tol uene for 1 h followed by rinsing with fresh toluene and quickly drying with N2. Curing o f the silane linkages was carried out in an oven at 100 °C for 2 h. At the end of the curing process, the substrates were removed from the oven and were allowed to cool to room te mperature.

Subsequently, PAA was deposited onto the APTES coated surfaces by spin coatin g. PAA solution was prepared by stirring 1 mg/ml and 2 mg/ml of PAA in methanol for 1 6 h and 15 min of sonication. Prior to the spin coating, the solution was filtered through a 0.2 micron Whatman® PFTE filter. Depending on the size of the substrate, a 10–50 µL dr op of solution was spread onto the substrate to fully cover surface and the sample was spi n-coated at 2000 RPM for 120 s. In order to improve the surface adhesion of the PAA cus hion, the substrates were baked to enhance amide formation between the PAA carboxylic acid and the amine functionality at the surface APTES layer.

2.4.2. Deposition of Supported Lipid Bilayers

Lipid bilayers were deposited onto the PAA coated substrate by the Langmuir-Blo dgett technique using a Teflon® Langmuir–Blodgett trough (Type 611, Nima, UK). The P AA coated substrate was first immersed in the subphase of Nanopure water while the tem perature of 18 °C was maintained by a water circulating system. A lipid monolayer was fo rmed by spreading chloroform solution of DMPC containing 1 mol% Texas Red® DHPE fluorescent probe at the air-water interface. After spreading, solvent was allowed to evapo rate for a minimum 15 min before the lipids at the air–water interface were compressed at

a rate of 10–15 cm^2/min to a lateral pressure of 45 Nm/m. Once the desired surface press ure was achieved, the monolayer was left to equilibrate and maintained at this pressure. S ubsequently, a monolayer was transferred to the substrate by vertically pulling the substra te through the air-water interface at a rate of 1 mm/min. After the substrate was fully rem oved from the subphase, the top leaflet was deposited by the Langmuir-Shaeffer method. In this case, the monolayer coated substrate was held horizontal to the air-water interface and transferred into the subphase at a faster rate, 4 mm/min, to prevent the lipids from dis persing back onto the air-water interface. After the second deposition, the substrate was pl aced into a submerged Petri dish and taken out of the trough while kept under water. Lastl y, the substrate was rinsed and soaked with different pH levels of buffer solution at low (1 0 mM) and high (140 mM) concentration of salt; citric acid-sodium citrate (pH 4), sodiu m-disodium phosphate (pH 7.4) and sodium carbonate-bicarbonate (pH 9.2). Throughout any changes, the membrane coated substrate was always maintained under water.

2.4.3. Fluorescence Recovery After Photobleaching (FRAP)

FRAP measurements were conducted on a Nikon Eclipse TE2000-S inverted fluor escence microscope (Technical Instruments, Burlingame, CA) equipped with two neutral density filters (ND8 and ND4), a Texas red filter set and a Retige-1300 CCD camera (Tec hnical Instruments). The fluorescent sample was placed in a small temperature controller on the microscope stage and was set to a temperature of 30 ± 1 °C. In addition, a thermoc ouple was fixed close to the sample in the region where the images were taken to verify t hat the lipid bilayer was above its transition phase temperature. A mercury lamp filtered t hrough the neutral filter was used to illuminate the sample. Images were taken using a Pla

n Fluor 10x (NA, 0.30) and were stored and processed using simple PCI software (Compi x, Inc., Cranberry Township, PA).

A typical FRAP measurement was performed by removing the neutral filters and b leaching a defined spot with a high power beam using a Plan Fluor 40x (NA, 0.60) for 15 0 sec, resulting in a 30–50 μm circular bleached spot on the fluorescent sample. To reduc e further bleaching of the fluorophore during the recovery period, the neutral filters were replaced and images were collected every 30 sec using the 10x objective in order to recor d wide-field images of the fluorescent recovery process. The series of images was used to calculate the diffusion coefficients.

The obtained videos were analyzed using a FRAP analysis program created in MA TLAB (MathWorks, Natick, MA) by the Parikh research team and is further described in detail elsewhere[86]. Briefly, for each frame of the bleached spot, the program tracks the pr ofile of the spot including the location, width and cross-sectional intensity, which can be approximated as a Gaussian function. Gaussian initial conditions extracted from the first post-bleached image are fit to a standard diffusion equation, Eq. 1. This produces a time-e volving Gaussian whose amplitude decays as time increases.

$$A(t) = \frac{C_o}{1 + \frac{2Dt}{\sigma_o^2}} \qquad \text{[Eq. 1]}$$

where C_o is the initial amplitude, t is elapsed time, σ_o is the initial width of the Gaussian a nd D is the calculated diffusion coefficient. Once the program fits the width and amplitud

e of the Gaussian curve at the corresponding elapsed time from the first post-bleached sp ot, the diffusion coefficient of the lipid bilayer can be determined using Eq 1.

An example output of the program is shown in Figure 2. The Gaussian amplitude curve as a function of time is fit to determine the diffusion coefficient.

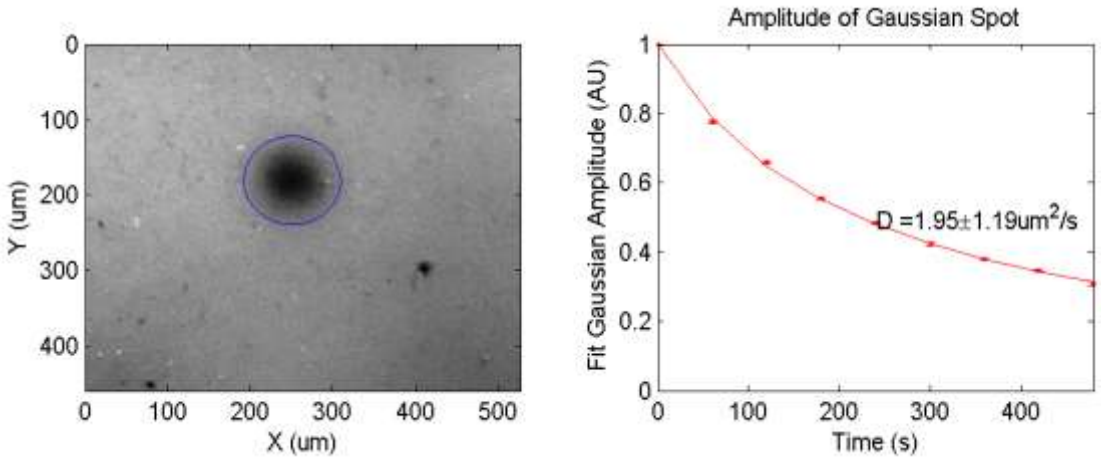

Figure 2. Output figures from the FRAP analysis program for DMPC doped with 1% Texas Red on 1 mg/ml concentration of PAA coated glass substrate at pH 7.4. The image on the left is the first post-bleached frame where the program analyzes the profile of the circled area. Next image shows the calculated diffusion coefficient by fitting the Gaussian amplitude at the corresponding elapsed time from the first post-bleached frame.

2.5 Results

Self-diffusion coefficients for DMPC lipid bilayers doped with 1 mol% Texas Red -DHPE supported on PAA coated glass prepared by Langmuir Blodgett were calculated u sing FRAP technique at different pH levels and PAA concentrations. Control measuremen ts of lipid diffusion on ultra-clean, UV-ozoned glass supports without PAA (uncoated) are provided for comparison. No difference was found if quartz or glass substrates were used for either the PAA or uncoated measurements.

Table 1: Diffusion coefficients of DMPC doped with 1 mol% Texas Red on glass substrates

	PAA concentration coated on substrate		
	Diffusion Coefficient [$\mu m^2/s$]		
pH	1 mg/ml	2 mg/ml	Uncoated
9.2	2.643 ± 0.795	2.529 ± 0.783	2.386 ± 0.332
7.4	1.925 ± 0.554	1.903 ± 0.856	1.726 ± 0.440
4.0	0.118 ± 0.049	0.099 ± 0.022	1.714 ± 0.150

For each system, over 10 different samples were prepared on different days and m easured over multiple locations (minimum of 4) to obtain the reported average diffusion c oefficient. Differences in the background intensities in the figures are due to small variati ons in the light intensity, optical alignment, and sample.

2.5.1. Influence of Starting PAA Film Thickness on Supported Lipid Bilayers Diffusion

In the first set of experiment, the influence of concentration of the PAA spin

coating solution on the diffusion coefficient was determined by studying fluorescent recovery in phospholipid bilayers supported on PAA coated glass or quartz substrates. Two different spin-coating solution concentrations were studied; 1 mg/ml and 2 mg/ml.

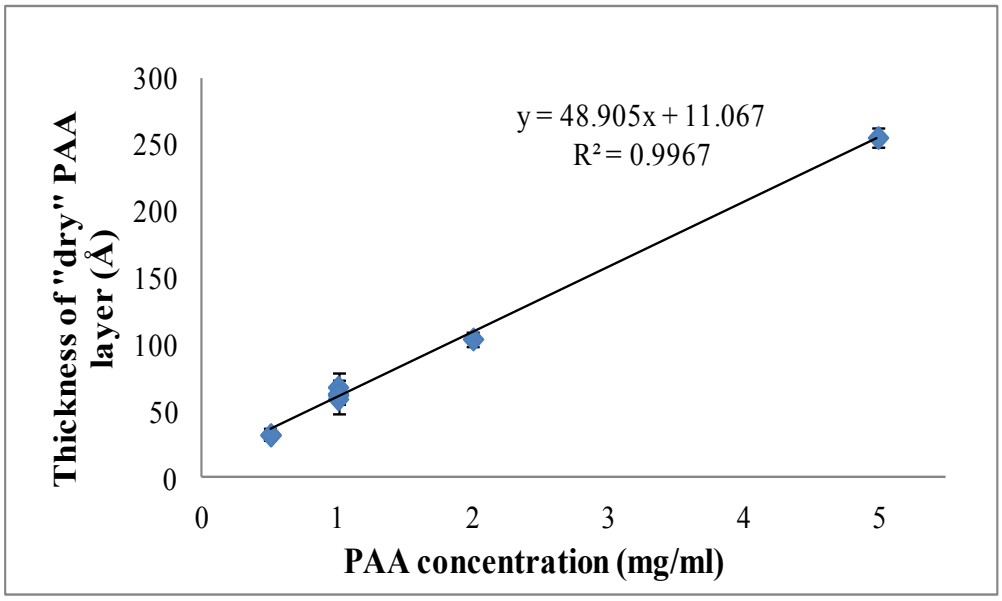

Figure 3. Thickness of the "dry" PAA cushion obtained from neutron reflectivity measurements conducted by Dr. Simon Castorph. As spin coating PAA concentration increases the thickness of the "dry" PAA layer linearly increases.

It has been previously reported that the deposited film thickness increases approximately linearly with solution concentration[85, 87]. Spin-coated PAA films were found to follow this linear behavior. The thickness of the "dry" PAA cushion as a function of the spin-coating concentration is shown in Figure 3. The neutron reflectivity measurements and analysis were conducted by Dr. Simon Castorph. Doubling the concentration of PAA solution from 1 mg/ml to 2 mg/ml approximately doubles the "dry" thickness of the PAA cushion.

Importantly, the diffusion of the lipid membrane atop the various PAA films was

found to be unaltered by the starting, “dry” PAA thickness. Because the thickness of the cushion can also be manipulated by pH, “dry” thickness is used here as a means to distinguish the effect of the overall amount of PAA cushion. At pH 7.4, the diffusion coefficients of a DMPC bilayer on PAA coated glass substrate at a PAA concentration of 1 mg/ml and 2 mg/ml were 1.93 $\mu m^2/s \pm 0.55$ ($N = 52$) and 1.90 $\mu m^2/s \pm 0.86$ ($N = 13$), respectively. Representative FRAP images of these are depicted in Figure 4 and Figure 5. As can be seen, the diffusion rate of the lipid bilayers in the two figures exhibits to be reasonably close.

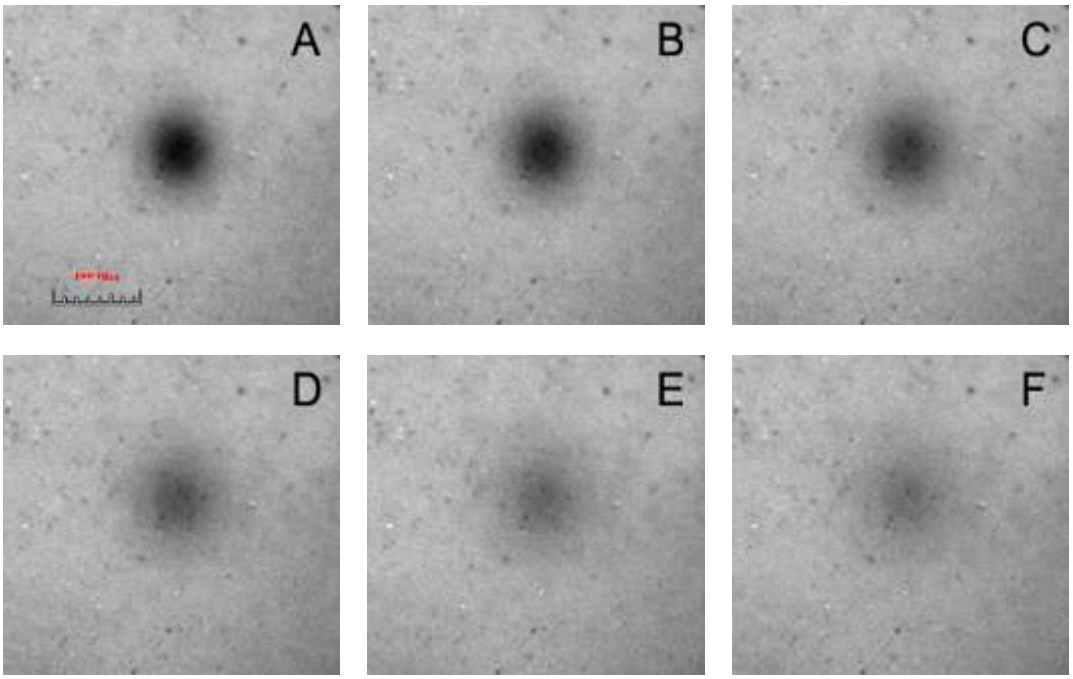

Figure 4. FRAP measurements of DMPC doped with 1% Texas Red on 1 mg/ml concentration of PAA coated glass substrate at pH 7.4. Image (A) was taken after 150 sec of photobleaching and the times elapsed from this point are (B) 30 sec (C): 1 min 30 sec (D): 3 min (E): 4 min 30sec (F): 6 min

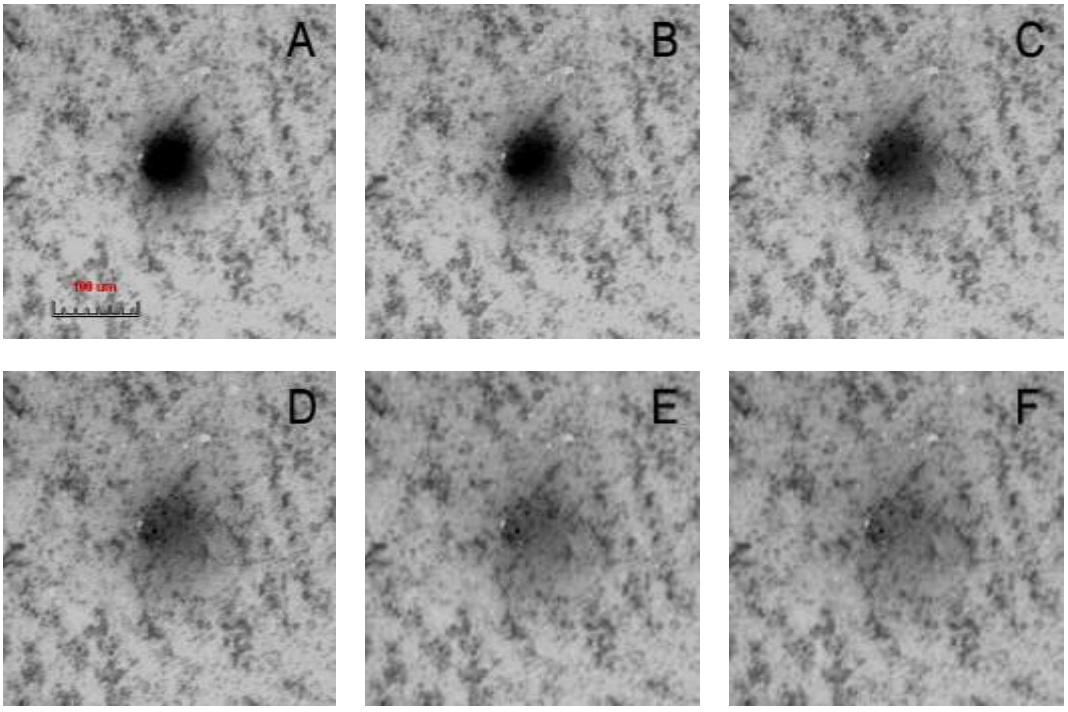

Figure 5. FRAP measurements of DMPC doped with 1% Texas Red on 2 mg/ml concentration of PAA coated glass substrate at pH 7.4. Image (A) was taken after 150 sec of photobleaching and the times elapsed from this point are (B) 30 sec (C): 1 min 30 sec (D): 3 min (E): 4 min 30sec (F): 6 min

According to t-test analysis, the evaluated P value from the two data set was 93%, which indicates the mean values of the diffusion coefficients are not significantly different. The P values at pH 9.2 and pH 4 were likewise high, verifying that membrane diffusion coefficients are unaltered by changing the starting thickness of the PAA cushion.

When working with polymer supported lipid bilayers, it is advantageous if their properties mimic the properties of freestanding bilayers as closely as possible. Indeed, it is ideal for the polymer film to have minimal interaction with the membrane to prevent any immobilization of the lipids or transmembrane proteins. In other words, the diffusion of the lipids on polymer supports should behave alike to the ones on the freestanding

bilayers. Surprisingly, our results revealed that in the absence of PAA cushion, the lipids exhibited a diffusion coefficient similar to the ones with PAA coated substrates except at low pH. At pH 7.4, the calculated average diffusion coefficient on a bare glass substrate was 1.73 $\mu m^2/s \pm 0.44$ ($N = 29$). This number is slightly lower than the PAA coated substrates nevertheless they all diffused within the same order of magnitude. In addition, the t-test result showed that diffusion coefficients on the PAA and bare substrates are not significantly different (P value = 10%).

2.5.2. Influence of the pH Level on PAA Supported Lipid Bilayers Diffusion

In the next set of experiments, mobility of the PAA supported bilayer was quantitatively measured at three different pH levels: pH 9.2, 7.4 and 4. Experiments were carried out with the same "dry" PAA film thickness using a 1mg/ml spin coating solution.

Previous investigations reported by other researchers have already measured the PAA polymer film thickness as a function of pH[82-83]. In particular, PAA has a pK_a of 4.6. Under alkaline conditions ($pH > pK_a$) the PAA chains become deprotonated and gradually expand or swell to 2–5 times the dry thickness. In contrast, as the pH decreases PAA film collapses because the degree of ionization of the carboxylic acid groups is small and it exist as –COOH instead. Therefore, the PAA thickness decreases to the degree of "dry" thickness under acidic conditions.

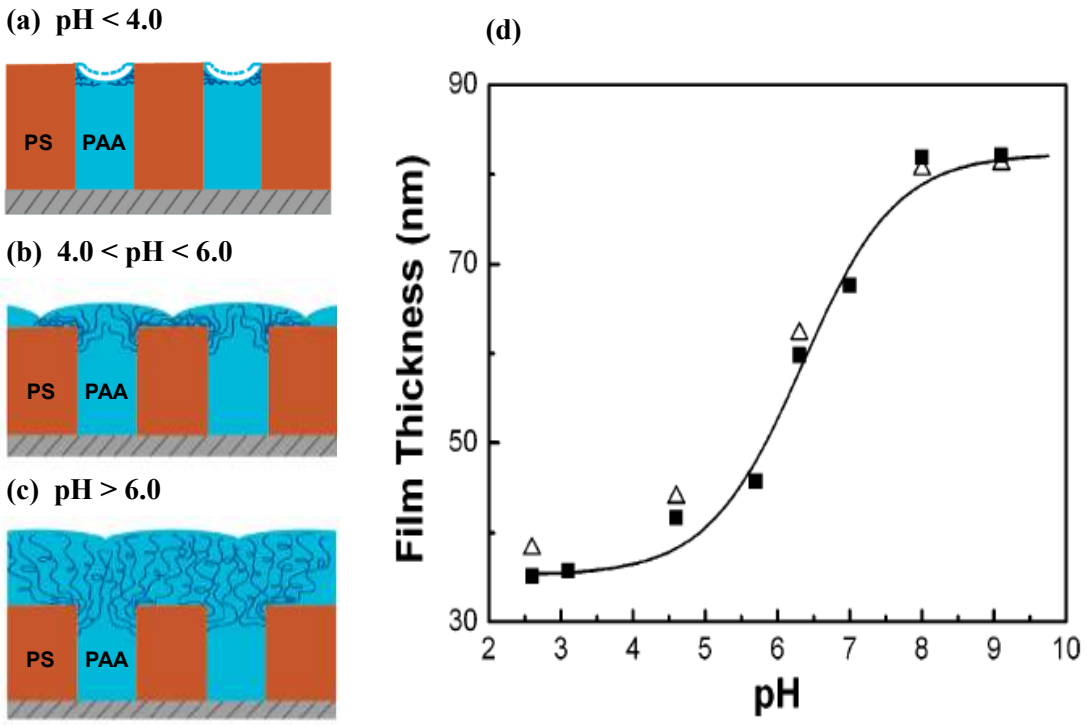

Figure 6. Schematic cartoons of perpendicularly aligned cylindrical PAA nanodomains embedded in a PS matrix at low, intermediate, and high pH regimes, where (a) pH < 4.0, (b) 4.0 < pH < 6.0, and (c) pH > 6.0, respectively. (d) Thickness of PS-*b*-PAA film on silicon substrates in aqueous media as a function of pH level. The "dry" thickness of the PS-*b*-PAA film is 33 nm, which is similar to the thickness at low pH regimes because PAA chains collapse within the cylindrical nanodomains. As the pH increases thickness of the film increases which is indicated as filled squares. In contrary, as the pH decreases the film collapses and overlaps suitably to the reverse swelling behavior which is shown as open triangles. Adapted from "pH-Responsive Nanostructures Assembled from Amphiphilic Block Copolymers" by Chen et al., 2006, *Macromol ecules*, 39, 6063-6070.

Fluorescence recovery after photobleaching (FRAP) data revealed that PAA cushioned bilayers are freely mobile when they were brought above their main gel-fluid phase transition temperature. Whereas, the transition temperature of DMPC is 24 °C[6], the conducted temperature was set at 30 °C. Above its transition temperature, the calculated diffusion coefficients were 1–3 $\mu m^2/s$ at pH 9.2 and pH 7.4 on either PAA coated or uncoated substrates, which are comparable to values typically reported for fluid bilayers supported on oxide substrates[61]. In contrast, the diffusivity was reduced by an order of

magnitude or more at pH 4 on PAA coated substrates. Figure 7 and Figure 8 demonstrates this point clearly. The fluorescence in the photobleached areas recovered completely in less than 10 min at pH 9.2 and pH 7.4 (see Figure 4) on 1 mg/ml or 2 mg/ml of PAA coated substrates. But at pH 4 the bleached spot would not recover fully until 30 min or more. It should be noted that the diffusion coefficient on the bare glass substrate was noticeably higher than the ones on PAA coated substrate at pH 4.

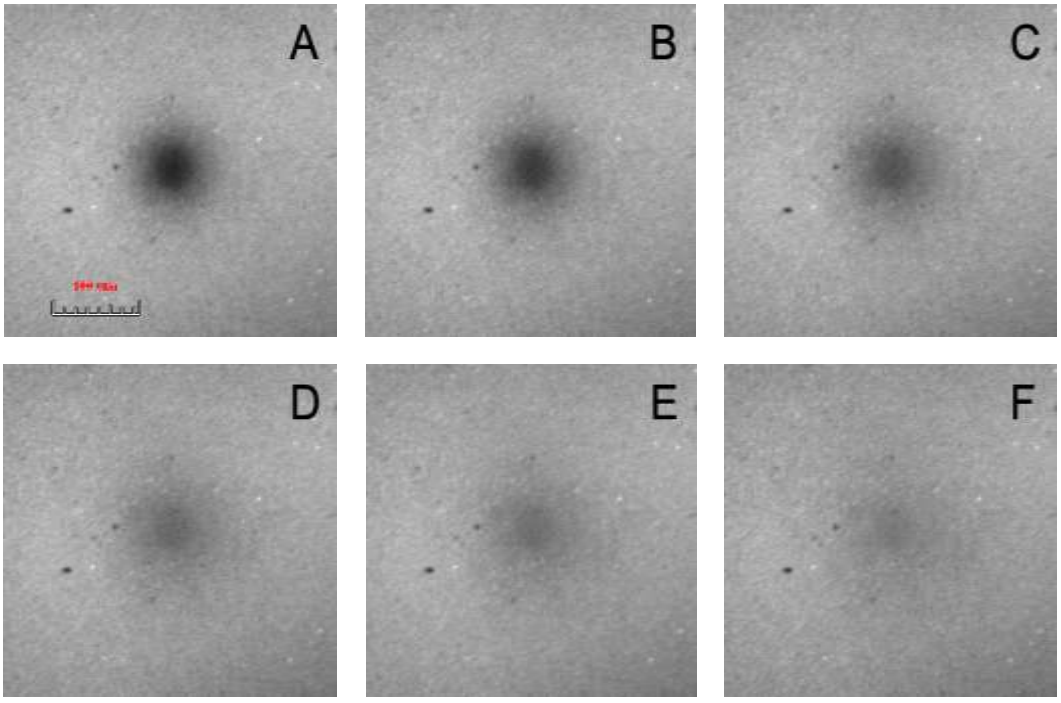

Figure 7. FRAP measurements of DMPC doped with 1% Texas Red on 1 mg/ml concentration of PAA coated glass substrate at pH 9.2. Image (A) was taken after 150 sec of photobleaching and the times elapsed from this point are (B) 30 sec (C): 1 min 30 sec (D): 3 min (E): 4 min 30sec (F): 6 min

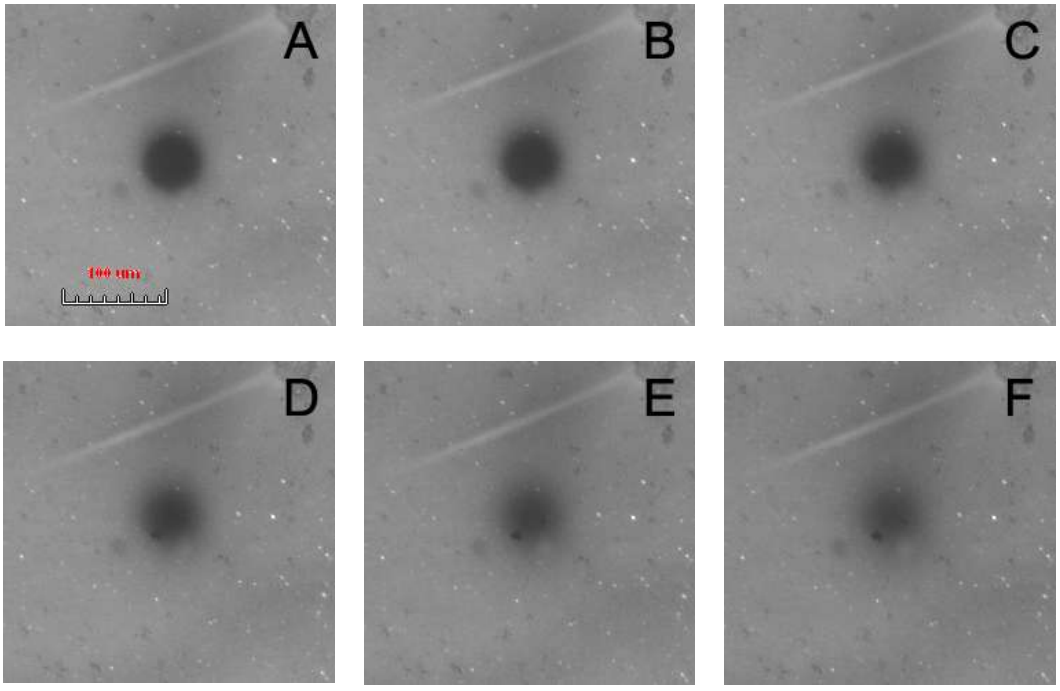

Figure 8. FRAP measurements of DMPC doped with 1 mol% Texas Red on 1mg/ml concentration of PAA coated glass substrate at pH 4. Image (A) was taken after 150 sec of photobleaching and the times elapsed from this point are (B) 30sec (C): 2min (D): 5min (E): 10min (F): 15min

To probe the suppressed rate of lipid diffusion on collapsed PAA cushions under acidic conditions, pH 4, the temperature of the FRAP measurements was increased. Remarkably, heating the samples up to 45 °C allowed the lipid bilayer to diffuse about the same rate as pH 9.2 or pH 7.4 (Not reported here), indicating that at lower pH level the transition temperature of gel-fluid phase is suppressed. This finding suggests that there is some kind of physical change to the lipid bilayer or coupling of the bilayer to the polymer cushion at lower pH levels.

2.5.3. Mobility of Phospholipids on PAA at Physiological Conditions

Human body contains about 140 mM of salt concentrations and maintains a pH

level of 7.4 in the blood stream[88]. To test the PAA supported lipid bilayer behavior under physiological conditions, we repeated the FRAP experiments on 1 mg/ml PAA solution and used 140 mM NaCl and 10mM pH 7.4 phosphate buffer solution for the medium.

The phospholipid bilayer supported on a PAA coated glass substrate had a homogeneous surface and an average diffusion coefficient of 1.92 $\mu m^2/s \pm 0.56$ ($N = 17$). This is almost identical to the value obtained at 10 mM phosphatebuffer solution that was reported in the previous section. In other words, increase in the monovalent Na^+ concentration had no effect on the mobility of the lipid bilayer. This result is in a good agreement with the conclusions of Jacobson and Papahadjopoulos[89]. They reported no appreciable effect of monovalent salt concentration on the phase transition temperature of electrically neutral PC bilayers. In contrast, the phase transition temperature of charged Dipalmitoylphosphatidylglycerol (DPPG) bilayers was found to increase as the ionic strength of NaCl increased.

2.6 Discussion

Reported diffusion coefficients by other authors for bilayers of DMPC on silicon wafers and POPC on quartz were 3–4 $\mu m^2/s$[22,61] which are 1–2 factors higher but in the same order of magnitude compare to the values measured here on glass substrates. Bilayers of POPC and DPPC on silica particles showed similar values[62-63].

In other studies, for a range of different solid inorganic supported substrates, the diffusion of lipids was shown to be somewhat reduced as compared to that of free layers. Diffusion coefficients found in water-swollen polyelectrolyte multilayer supported lipid layers were on the order of 10^{-3} $\mu m^2/s$[60]. For lipid monolayers and bilayers deposited onto polyacrylamide gel with various compositions, diffusion coefficients in water varied in range of 10^{-2}–10^{1} $\mu m^2/s$[66]. Here, in contrast to earlier works, phosphotidylcholine lipid bilayers were shown to diffuse significantly faster when supported on a PAA cushion. Indeed, PAA supported membranes diffused slightly faster than membranes supported on hydrophilic glass.

Using FRAP experiments, this work has shown that DMPC bilayers can diffuse efficiently on glass substrates that have been coated with PAA. In addition, no effect of the starting, “dry” thickness of the PAA cushion was shown. Thus, the total thickness of the cushion can be easily adjusted to meets the needs of different applications. On the other hand, expanding/collapsing the PAA cushion by changes in pH of the aqueous solution had a substantial effect on supported membrane lipid diffusion. Specifically, under alkaline and neutral conditions when the cushion was swelled, DMPC bilayers had

lipid diffusivities similar to those measured on bare glass. In contrast, under acidic conditions when the cushion was collapsed, the diffusivity was greatly diminished and slow fluorescent intensity recovery was observed. This change can be explained in terms of increase of phase transition temperature of the lipid bilayer due to stronger interactions with protonated carboxylic acid groups. The pKa value of the amine group in pure DMPC membrane is ~7.9[90]. Thus, there shouldn't be a change in its charge for these pH ranges. Therefore, the alteration is due to a change in the interaction of the lipid with collapsed, protonated PAA, which led to a depression in the mobility of the lipid bilayer.

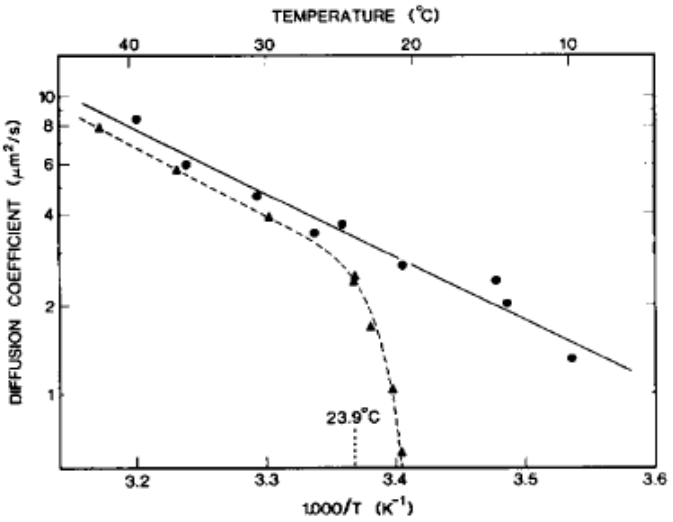

Figure 9. Lateral diffusion coefficients of the fluorescent lipid probe NBD-PE (0.5 mol%) in single DOPC (●) and DMPC (▲) bilayers supported on silicon wafer. Adapted from "Supported Phospholipid Bilayers" by Tamm and McConnell, 1985, *Biophys. J.*, 47, 105-113.

As noted above the effect of temperature on diffusion can be striking. Because viscosi ty is highly dependent on temperature, it affects the mobility of both soluble membrane and membrane-associated molecules. Other papers have discussed about this point in detail[6,59]. At any temperature above their chain-melting phase transition temperature, diffusion is fast. Even a slight change in the temperature can drastically affect the diffusion coefficient especially around the phase transition temperature.

The change in membrane diffusivity by pH modification of the PAA cushion provides evidence that the bilayer is more coupled to the underlying cushion layer under acidic conditions. Importantly, this simple experiment demonstrates that by changing the pH of the surrounding medium of the PAA cushion it is possible to modulate membrane-cushion interactions and quantify the effect on membrane properties.

2.7 Conclusion

The extreme sensitivity of bilayer dynamics to multiple parameters can explain the great variety of experimental results found in the literature. The extensive study presented here, based on the diffusion coefficient measurements of phospholipids on PAA using a fluorescence recovery technique, highlights some important results. Diffusion experiments on PAA supported lipid bilayers were performed as a function of PAA "dry" thickness and pH of the surrounding medium. To this end, the "dry" thickness of PAA does not affect the diffusion coefficient of lipid bilayer. However, the FRAP experiments clearly demonstrate that the pH level of the aqueous solution strongly affects the lateral motion of phospholipids on PAA coated substrates. Herein, restricted lateral diffusion of supported membranes was achieved under acidic conditions when the PAA cushion is collapsed and the carboxylic acid groups are protonated, presumably because of an increase in the lipid phase transition temperature due to interactions with the underlying PAA cushion. This demonstrates that pH modifies membrane-cushion properties, through changes in the PAA cushion's three-dimensional topology and coupling of the membrane to the cushion. Furthermore, under physiological or alkaline conditions PAA supported lipid membranes diffuse as fast as free bilayers on bare, hydrophilic glass substrates.

These results bring new insights into the potentials of PAA as a platform for supported membrane systems. In particular, the system should be amenable for providing a 2-dimensional membrane system that closely mimics free-standing membranes for elucidation of membrane properties and membrane-protein interactions, as well as provide a framework for studying integral membrane proteins where the influence of the

underlying cushion is minimized. By varying the pH level and PAA “dry” film thickness, the properties of the system can be tailored for different applications. From a biomimetic point of view, subsequent studies can be expanded to more complex membrane compositions and the incorporation of various proteins. Finally, simple amide coupling reactions with available carboxylic acid groups from PAA provide a convenient means to functionalize the cushion with biomolecules of interest to enable a variety of membrane function and interaction studies in future studies.

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