8-2010

Formation and Characterization of Polymerized Supported Phospholipid Bilayers and the in vitro Interactions of Macrophages and Fibroblasts.

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To the Graduate Council:

I am submitting herewith a thesis written by Jonathan Michael Page entitled "Formation and Characterization of Polymerized Supported Phospholipid Bilayers and the in vitro Interactions of Macrophages and Fibroblasts.." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Polymer Engineering.

Wei He, Major Professor

We have read this thesis and recommend its acceptance:

Roberto Benson, Tim Sparer

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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Formation and Characterization of Polymerized Supported Phospholipid Bilayers and the *in vitro* Interactions of Macrophages and Fibroblasts

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Jonathan Michael Page

August 2010

DEDICATIONS

I dedicate my master's thesis to my wonderful wife, parents, and family. I have been truly blessed that the people closest to me have always supported all of my endeavors and continue to support me in all that I do.

ACKNOWLEDGEMENTS

I am very grateful to everyone who helped me complete this thesis. I would especially like to thank Dr. Wei He who has always offered helpful insight and direction. I would to thank Dr. Roberto Benson for introducing me to this field and always focusing on my education and personal development. I would like to acknowledge Dr. Tim Sparer for his support with the cell culture work and guidance. I would also like to acknowledge Dr. Scott Saavedra and his group for providing me with the raw materials and very helpful discussions. Finally I would like to thank all of my friends and colleagues. Their never ending support and friendship provided me with the mental fortitude to complete this research.

ABSTRACT

Planar supported, polymerized phospholipid bilayers (PPBs) composed of 1,2-bis[10-(2',4'-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphocholine (bis-SorbPC or BSPC) were generated by a redox polymerization method. The PPBs were supported by a silicon substrate. The PPBs were characterized and tested for uniformity and stability under physiological conditions. The PPBs were analyzed *in vitro* with murine derived cells that are pertinent to the host response. Cellular attachment and phenotypic changes in RAW 264.7 macrophages and NIH 3T3 fibroblasts were investigated on PPBs and compared to bare silicon controls. Fluorescent and SEM images were used to observe cellular attachment and changes in cellular behavior. The PPBs showed much lower cellular adhesion for both cell lines than bare silicon controls. Of the cells that attached to the PPBs, a very low percentage showed the same morphological expressions as seen on the controls. The hypothesis generated from this work is that defects in the PPBs mediated the cellular attachment and morphological changes that were observed. Finally, a layer-by-layer (LbL) deposition of a poly(acrylic acid) (PAA) and poly(N-vinylpyrrolidone) (PNVP) alternating bilayer was attempted as a proof of concept for future modification of this system.

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CHAPTER 1

1.1 Background and Literature Review

1.1.1 Host-Material Interactions

In the field of biomaterials, the interaction between the immune system and the surface of materials is of great importance. All biomaterials that are inserted into the body initially stimulate the immune system and the generalized host response [1-3]. This response correlates with the degree of injury, the type of tissue, and the surface chemistry of the material [4-5]. The interaction can be beneficial, harmful, or utilized by a bioengineer to generate a desired outcome. The most important factor that mediates how the host system reacts to biomaterial surfaces is the adhesion of proteins [1-3, 6-7].

The first interaction a host body has with any foreign object is the adsorption of blood serum proteins onto the surface of the material [3, 7-9]. This is directly followed by the formation of a provisional matrix, mainly the fibrin and fibrinogen thrombus, that surrounds the material [1-2, 6]. The cascades that are generated by the clot formation can cause a spontaneous desorption and adsorption of proteins that have a higher binding affinity for the materials surface. This mechanism is driven by a well known phenomenon, the Vroman effect [2]. The types of proteins that preferentially adsorb to the materials surface can directly affect how the immune cells behave [5, 10]. Fig. 1 displays the host response in regards to foreign materials. Typically the first cells that follow material implantation are polymorphonuclear leukocytes (PMNs) such as neutrophils [2]. The presence of these cells is associated with the acute stage of

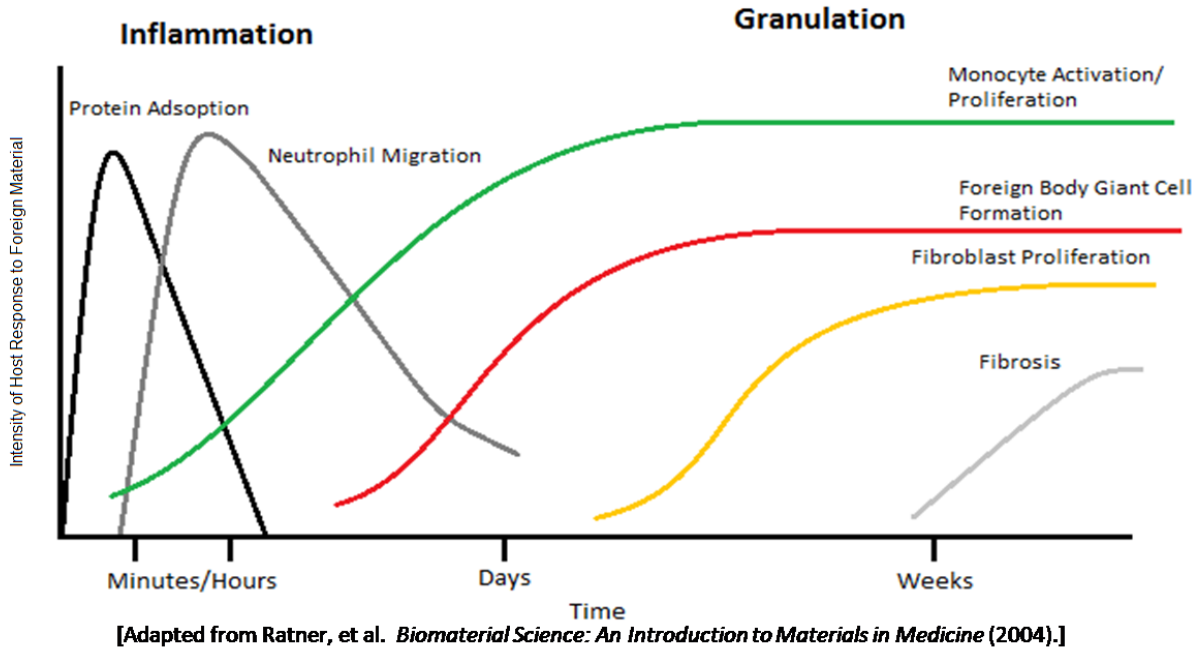


Figure 1. Diagram of the host response to foreign material over time.

inflammation that generally only lasts for a week [2]. The PMNs release specific chemoattractants that recruit phagocytes and aid their adhesion [2]. The infiltration of phagocytic cells, mononuclear monocytes/macrophages, is associated with the late stage of inflammation. This stage can be short lived, lasting a few days to two weeks for biocompatible materials [1-2, 10].

The final stage of the host response is the granulation and neo-vascularization stage [2, 11]. This stage is associated with high macrophage, fibroblast, and foreign body giant cell populations [11]. These cells facilitate the formation of a fibrous capsule around the implant to isolate the material [11]. If the material can be degraded by the body, then eventually this fibrous encapsulation can be remodeled into neo-tissue. However, if the material is non-degradable, then a state of frustrated phagocytosis is activated [2]. This stage results in the fibrous capsule separating the material indefinitely. Macrophages and fibroblasts play a key role in the chronic and granulation stages of wound healing [10, 12]. These immune cells typically do not interact with the biomaterial surface itself, but with this layer of adsorbed proteins. Therefore if one can prevent the fouling of a biomaterials surface with proteins, the interactions with the host immune system will be greatly diminished.

A successful method of mediating the protein adsorption of a biomaterial, which in turn limits the negative host response, is the utilization of a non-fouling surface modification. The purpose of this thesis is to study a particular biomimetic, non-fouling surface modification and its effects on the adhesion and behavior of key cellular components in the host response system.

1.1.2 Thermodynamics of Protein Adsorption

As mentioned above, the most important interface of materials and a host system is the protein and surface interaction. However, to fully understand how to prevent or limit this interaction, one must begin to understand the mechanism of protein adsorption. The thermodynamics of protein adsorption onto any surface is based on the simple application of the Gibbs free energy equation, shown in eq. 1 [13].

$$\Delta G_{ads} = \Delta H_{ads} - T\Delta S_{ads}$$

where Δ_{ads} is the change in free energy, G, enthalpy, H, and entropy, S, caused by the adsorption phenomenon.

This process is spontaneous for most situations with ΔG_{ads} being less than zero. Early studies by Norde and Lyklema found, by studying the adsorption of proteins on polystyrene surfaces, that this process is entropically driven, or dependent on the interaction of the surface and the protein with water [13]. This process of water displacement and protein adsorption is shown in Fig. 2. The important finding from this study was that hydrophobic surfaces have a stronger association with proteins than hydrophilic surfaces [13]. As the surface is more hydrophilic there is a stronger affinity of water, which translates to a larger entropic penalty by disassociating the bound water upon adsorption of proteins.

It is also highly important to mention that structure of the adsorbed protein, or whether or not the protein maintains it native configuration, can be affected by charge density, ionic strength, and intramolecular hydrophobic interactions. Specifically, the hydrophobic interactions, which protect the secondary structures of common globular proteins, will

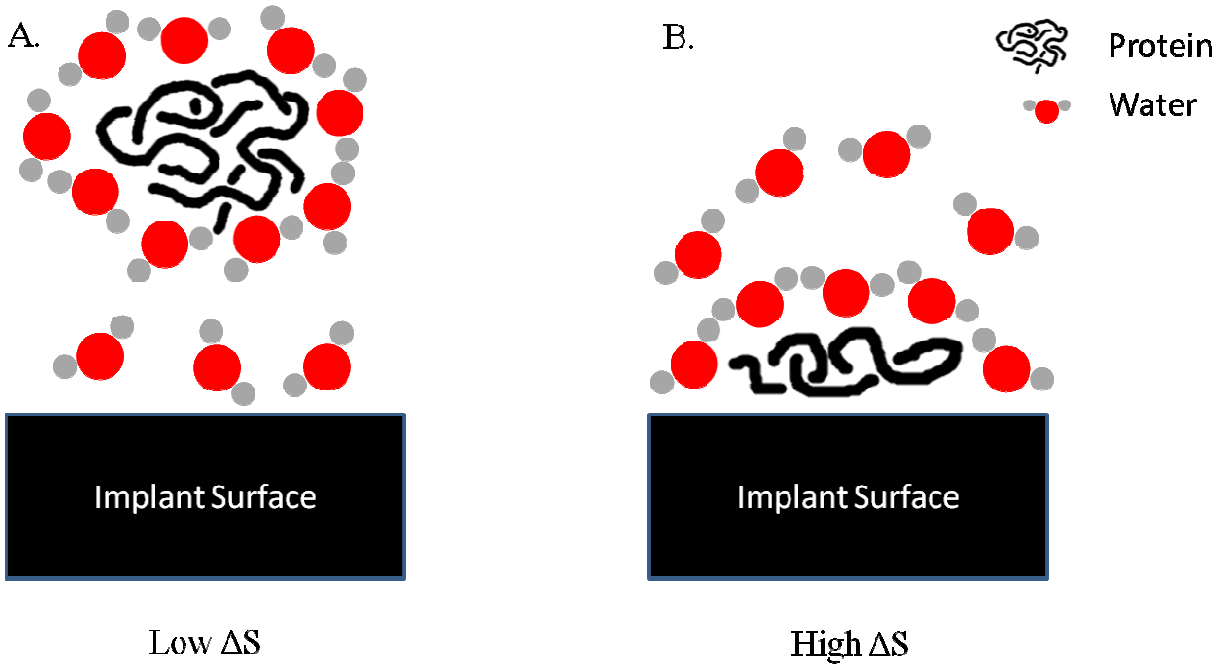


Figure 2. Depiction of protein interactions with the surfaces of materials. The loss of bound water molecules, from both the protein and the surface, causes the gain in entropy and makes this process thermodynamically favorable.

readily interact with hydrophobic surfaces [13]. This causes the protein to denature and also increases the entropy of the system, making the process spontaneous. The reconformational process of proteins was found to be an enthalpic process that was endothermic, but generally, unless large amounts of ions are transported from the water to the protein layer, this process cannot overtake the entropic contributions of the interactions with water [13]. Thus, the protein remains denatured and cannot return to its native state. The denatured proteins allow the host system to recognize a non-native structure, which begins the cascade of the biological response. While this work was thorough in defining the thermodynamic processes during protein adsorption, this was simply a generalized theory that shows promise in describing particular phenomenological events. As with all models however, the real system is far more complex. However, the notion that hydrophilic substrates prevent protein adhesion, lead to the first step in synthesizing non-fouling surface modifications.

1.1.3 Non-Fouling Surface Modifications

A surface can be defined as non-fouling if there is little to no protein adsorption and/or cellular attachment. These modifications are greatly significant in the field of biomaterials. The first attempts to generate non-fouling surface was influenced by the requirement of surface modifications to lower protein interactions. This was accomplished by several methods, such as immobilization of proteins [14-16], hydrophilic polymer tethering [12, 17-18], or graft copolymerization with hydrophilic monomers [19-20]. Table 1 shows a compiled list of popular non-fouling surface modifications. The immobilization of proteins onto surfaces was pioneered for the use in assay technology in the burgeoning analytical medical fields. These assays were utilized to quickly and cheaply test blood banks for infectious diseases. This use prompted others

Table 1. Compiled list of popular non-fouling surface modifications and some corresponding publications.

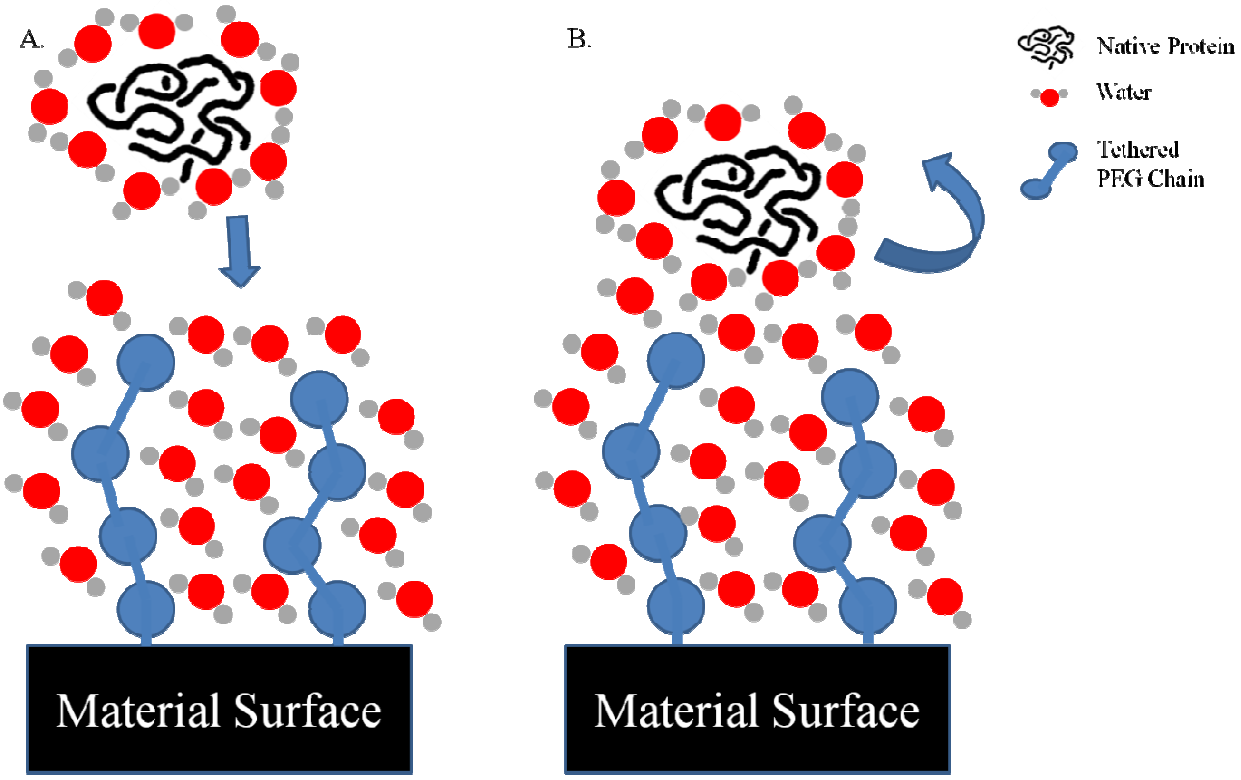
Surface Treatment	Advantages	Disadvantages	Publications
PEG-Grafted Surfaces (10 nm -100 µm thickness)	• Non-toxic • Covalently tethered to surface • Extremely hydrophilic	• Grafting density and Mw changes resistance • Degradable over time by hydrolysis	[21-22]
Phospholipid copolymers (100 nm – 100 µm thickness)	• Polymer composition can be engineered for specific applications • Multiple methods of utilization (adsorbed onto surface, covalently tethered, bulk polymer substrates, etc.)	• Possible toxicity issues of degradation products • Density of exposed phospholipid is lower than SAMs	[23-25]
Self Assembled Monolayers (SAMs) (5 nm – 50 nm thickness)	• Variable and tunable chemistry • Density is increased due to self packing	• Limited to specific surfaces (mainly metals) • Stability issues in physiological conditions	[26-28]

to look into the immobilization of proteins to prevent immune reactions towards materials. The early work of Kiaei et al. associated the adsorption and immobilization of albumin, a common serum protein that does not cause an immunogenic response when adsorbed onto biomaterial surface, with the prevention of further protein fouling [15]. This was accomplished by pre-treating the surfaces with glow-discharge plasma. This would generate a surface that could physically adsorb proteins with a much higher affinity than that of non-treated surfaces. They had found that other serum protein adsorption was greatly reduced when albumin was immobilized [15]. However, immobilization of proteins was not a full solution, due to the aforementioned Vroman Effect. This caused the preferential desorption and replacement of physically adsorbed proteins with higher affinity proteins. This led other researchers to try tethering the proteins to poly(ethylene glycol) (PEG) spacers to minimize surface interactions [29]. This approach showed some promise, but also ran into similar issues with competition of modified and non-modified protein adsorption. The difficulties resulting from these studies led researchers to focus mainly on synthetic molecules or polymers covalently attached to surfaces to prevent protein fouling.

The beneficial properties of hydrophilic non-ionic surfaces in preventing protein fouling had become quite apparent during this time. Many groups began simultaneously developing similarly designed surface modifications. The gold standard quickly became poly(ethylene oxide) PEO or PEG immobilization, grafting, or copolymerization. Early work by Jeon et al. and Desai and Hoffman provided the catalyst for the growing amount of research on PEO as a non-fouling surface [17, 30-31]. Desai and Hoffman completed a study of hydrophilic polymers

adsorbed onto polyurethane substrates. They determined that there was a molecular weight dependency on the adsorption of proteins and cellular components [17]. Chain mobility and hydrophilicity were hypothesized as the mediating factor of adsorption. Jeon et al. further expanded on the theoretical understanding of why PEO prevented protein adsorption. This group utilized a PEO containing block copolymer adsorbed to hydrophobic surfaces as a model to describe the observed phenomenon of low protein adhesion [30-31]. This model assumed that the PEO was terminally attached and the protein was of infinite size [30-31]. They found that there was a steric repulsion of the polymer chains that aided in preventing proteins from interacting with the underlying surface. The water soluble polymer chains were extended into a brush conformation and if a protein tried to reach the surface there would be a compression force expressed onto the polymer interface [30-31]. This force would greatly outweigh the van der Waals attraction and hydrophobic interactions of the protein and the underlying surface. The conclusion followed that a high surface density and long chain length was required for protein resistance [30-31]. The generalized model for this system is shown in Fig. 3.

This theory was tested by Prime and Whitesides with their utilization of self assembled monolayers (SAMs) of thiolated oligo(ethylene oxide) (OEG) of varying lengths to analyze the adsorption of various proteins and enzymes [27]. They determined, based on the adsorption curves of the proteins, that there was a non-specific resistance that was a factor of the hydrophilicity of the surface and the molecular weight of the OEG SAMs [27]. The findings concluded that short units of ethylene oxide could also prevent the adsorption of proteins [27]. They associated this difference from previously tested systems by the increased packing density of SAMs. The coverage of the surface was directly proportional to the resistance of proteins [27].



[Adapted from Morra, M. (2000) *On the molecular basis of fouling resistance.* J. Biomater. Sci. 11(6): 547-569]

Figure 3. Generalized model of protein interactions with PEG tethered surfaces. As the protein approaches the surface (A), the combination of steric hindrance of the tethered polymer chain and the solvation shell of water prevents long term protein adsorption and denaturing (B).

Based on these studies an attempt was made by A. Halperin to generate a better theoretical model of protein-interface interactions. This theory combined the steric hindrance models along protein size and a better thermodynamic interpretation of the repulsive forces attributed to the polymer brushes [22]. This work furthered the theoretical interpretations, but there was at least one major problem with this model. It neglected the chemical interactions with water. However, this was adjusted by incorporating the dynamic interactions of hydrogen bonding. Work by Wang et al. and Harder et al. that determined that the conformation of OEG SAMs greatly affects the protein adsorption [32-33]. The confirmation is a function of the molecular weight and is affected by hydrogen bonding with water [32-33]. After years of research the complex understanding of protein resistance can be attributed to entropic penalties from conformational hydration and the steric hindrances of polymer compression.

Although there was a large deal of advances made in the theoretical understanding of PEO based protein resistance, this system still has problems. It has been shown that PEO loses some of its non-fouling properties and exhibits oxidative degradation in physiological conditions [34-35]. This led researchers to look elsewhere for non-fouling surface modifications. A large number of alternative systems were found to exhibit non-fouling characteristics. Many of these systems took inspiration from biological materials.

1.1.4 Biomimetic Surface Modifications

Bioengineers have been attempting to mimic native biological structures even before the inception of modern medicine [36]. Fig. 4 shows the timeline of biomimetic materials utilized in

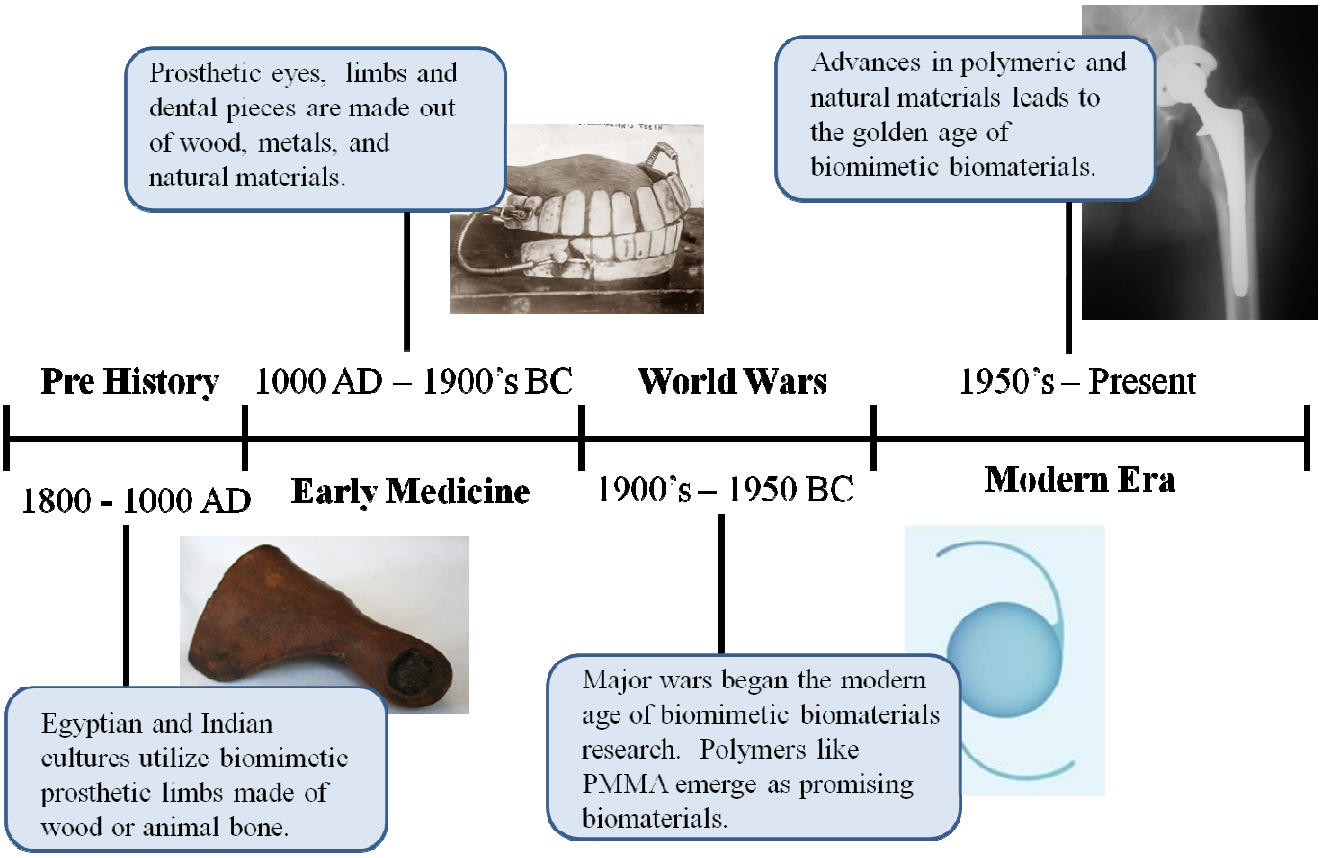


Figure 4. Timeline of biomimetic biomaterials in human history. This figure marks the transition of materials and biomaterial engineering throughout modern history.

medicine. Current trends in biomimetic materials span the gambit from osteogenic engineering, neural regeneration, biosensors, tissue engineering, peptide assembly, and non-fouling surfaces.

Biomimetic non-fouling surfaces, designed to resist protein adsorption and cellular adhesion, have been prepared and characterized from a large number of systems, including polysaccharides [37], peptides [38], and phospholipids [24]. The key factors of these systems are utilization of natural or mimicking materials to induce biocompatibility, hydrophilicity, and prevention of non-specific protein adsorption and cellular attachment.

Polysaccharides are utilized as non-fouling surfaces due to biocompatibility and their innate role in biology as stabilizers and compatiblizers [39-40]. The unique chemical structures of some biologically derived polysaccharides make them ideal components for surface modifications [37]. Polysaccharides such as alginic acid (AA) and hyaluronic acid (HA), chemical structures shown in Fig. 5 B-C, have been studied as non-fouling materials [37]. The success of these materials comes from their interactions with water. Both of these natural polymers have distinct ability to form dynamic bonds with water. Studies by Morra and Cassineli demonstrate that both AA and HA show distinct non-fouling properties when adsorbed to glass slides [37]. In these cases homopolymers are ionically bound to substrates by a poly(ethylene imine) (PEI) binder, shown in Fig. 5 A. This leads to a linked layer, shown in Fig. 5 D, quite different from the PEO brushes discussed earlier. While it is assumed in the PEO system that the steric hindrances cause some if not most of the non-fouling properties, a slightly different mechanism was elucidated for the polysaccharide systems. It was concluded that the hydration layer that is dynamically adjusting to better interact

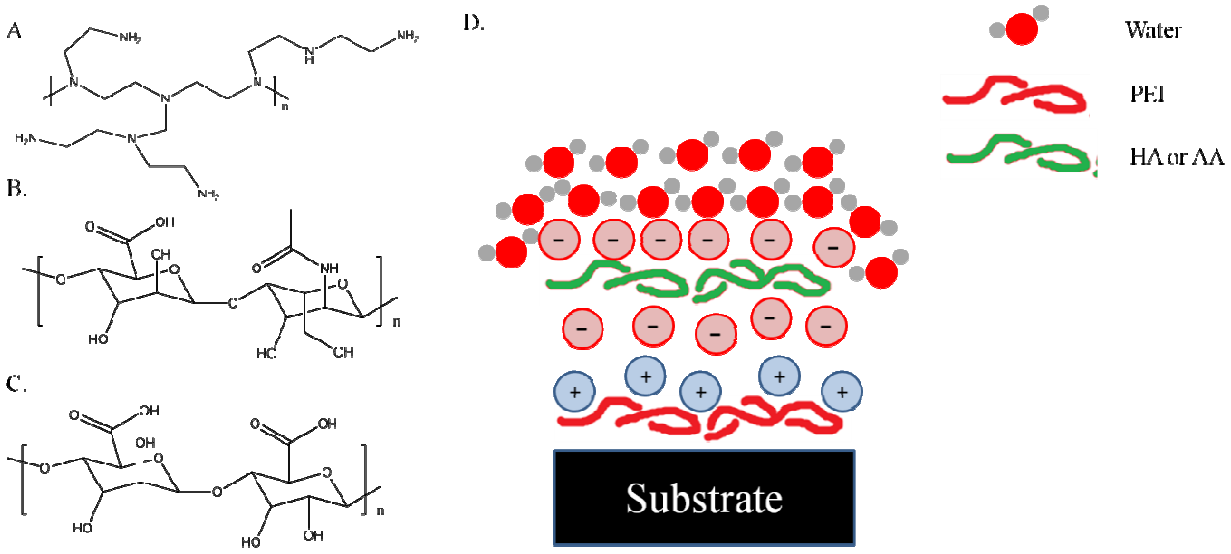


Figure 5. Chemical structures of PEI (A), HA (B), and AA (C), as well as a diagram of the electrostatic interactions of the positively charged PEI binding layer (red) with the negatively charged polysaccharide (green) (D). The hydration layer is also shown as a tightly bound layer of water molecules.

with the polysaccharide layer causes the protective properties seen experimentally [37]. However, these materials held net charges that could attract oppositely charged proteins. The relationship between charge and fouling has been a focus of much research. The properties of native polysaccharide systems were matched by researchers studying natural, charged peptide assemblies.

Peptides are assembled in biological systems to perform any number of complex tasks and functions. It was a logical step to analyze the behavior of synthetically assembled peptides that match the requirements of a non-fouling surface. Chen et al. utilized charged amino acids to synthesize a thiol terminated SAM precursor [38]. The authors synthesized random copolypeptides of net negative charged amino acids, glutamic acid (E) and aspartic acid (D), and net positive charged amino acids, lysine (K) and arginine (R). They found that the combination of EK and DK provided the most resistant surface when examining protein adsorption [38]. The lack of resistance with arginine based copolypeptides was thought to be caused by the weakly hydrated guanidinium unit [38]. The E and K residues are also found on the outer surface of albumin, and the authors note that the protein resistant behavior of monolayers of albumin could be associated with the charge balance of these moieties [38]. They conclude that the hydration shell provided by the protonated and deprotonated amino acids and the charge balance causes the extremely low adsorption of proteins onto these surfaces [38]. The notion that charge affects protein adsorption was shown in early works by Horbett and Hoffman [41]. This idea, that charge affects protein adsorption, lead researchers to analyze biomimetic zwitterionic molecules [42]. These molecules have both a positive and negative charge, but a net charge of zero. One of the most successful applications of biomimetic zwitterions is phospholipid based systems.

1.1.5 Phospholipid Surface Modifications

Phospholipids, which comprise a large majority of mammalian cell membranes, have been widely utilized as non-fouling surface modifications [43-44]. The chemical structures of phospholipids have two components, a hydrophobic alkyl tail and a polar hydrophilic headgroup, shown in Fig. 6 A [43, 45]. The polar headgroups could have a net negative, positive or neutral charge. Fig. 6 B displays the phospholipid components of cell membranes in human red blood cells and Fig. 6 C displays some common headgroup chemical structures of phospholipids [43-44, 46]. The asymmetry seen between the inner and outer leaflets of most cell walls allows for specific functionality of the cells. The zwitterionic phospholipid phosphatidyl choline (PC) is found in large quantities in the outer leaflets of cell membranes [43, 46].

In order to better understand the current project a brief introduction on the current state of research in phospholipid surface modifications will be covered. Generally, there are three methods of applying phospholipids as surface modifications: (1) bulk modified polymers [23-25, 47-48] (2) supported lipid bilayers (SPBs) [49-51] (3) polymerizable phospholipid bilayers (PPBs) [52-57]. Ishihara et al. produced a large volume of research on the bulk modified polymer 2-methacryloyloxyethyl phosphorylcholine (MPC) [24]. The MPC system has been successfully applied to many biomaterials, illustrated in table 2. This PC based acrylic polymer, chemical structure shown in Fig. 7 A and self assembled configuration shown in Fig. 7 B, can be utilized as a hydrogel or polymeric coating on many materials [24]. It was found to have significantly lower adsorption of proteins and cellular attachment compared to many traditional non-fouling systems [25]. Over the course of several years, this group conducted research as to

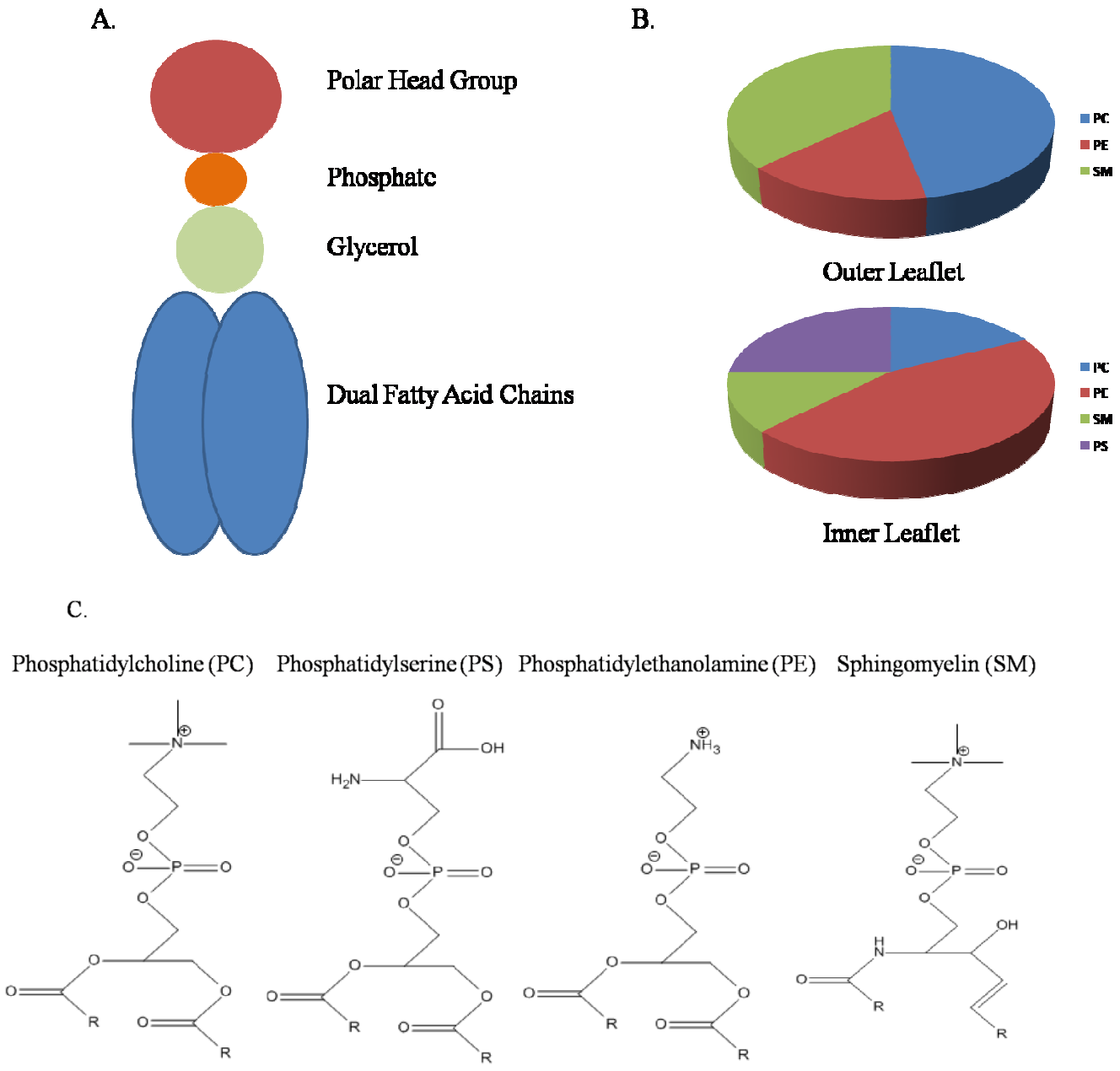


Figure 6. Generalized diagram of phospholipids (A), phospholipid composition of the inner and outer leaflets of red blood cell membrane (B), and chemical structures of some common phospholipid molecules, where R = alkyl chains (C).

Table 2. Applications of the phospholipid polymer MPC in biomaterials.

Application	Publications
Coating for Artificial Hip	[58]
Catheter Coating	[59]
Biosensor Coating	[47, 60]
Contact Lens	[61-62]

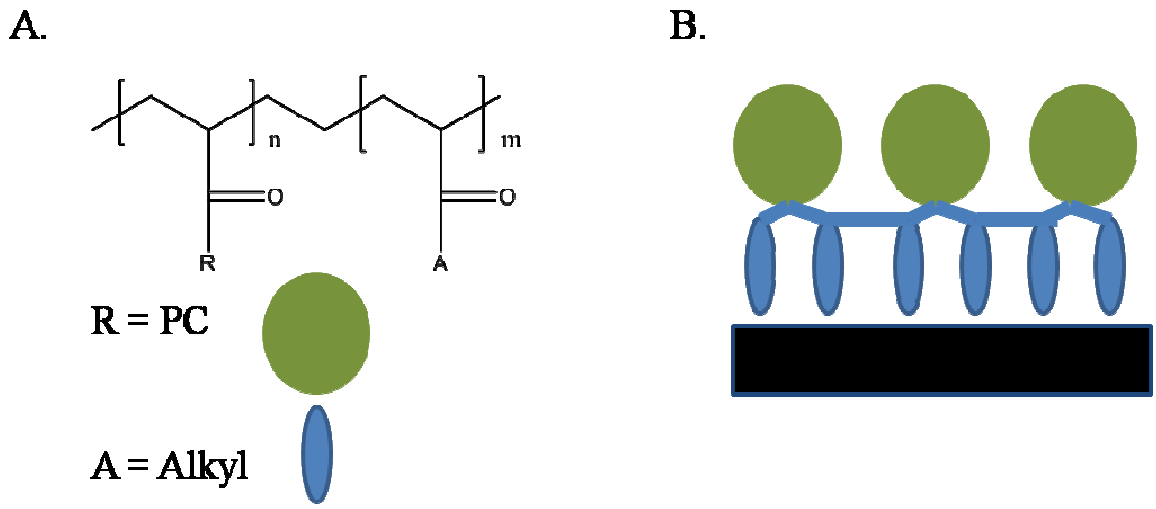


Figure 7. Chemical structure of MPC (A), self assembled structure of MPC copolymer when deposited onto a hydrophobic substrate (B).

what contributed to the unique property of this system. They found that when applied to a bulk system in an aqueous environment the PC headgroups will preferentially assemble along the interface with water [25]. This generates a solvation shell of strongly bound water. The water can form hydrogen bonds to the PC as well as ionic interactions [25]. They concluded that the large volume of water dynamically bound to the PC headgroups greatly limits the interactions of the proteins with the underlying surface [25]. It was also found that cellular attachment and activation was greatly limited, most certainly due to the lack of protein adsorption [23]. The MPC system has been applied to a large volume of systems from catheter tubes to contact lenses and has had a large degree of clinical success. However, the packing density of PC headgroups cannot match that of native, self assembled lipid membranes. Thus, several groups began to study the self assembled nanosystems of SPBs.

SPBs can be readily formed on nearly any surface that is relatively flat and hydrophilic. The self assembly mechanism, shown in Fig. 8, is driven by the hydrophobic/hydrophilic, electrostatic, and van der Waals interactions of the lipid molecules with the surface [45]. SPBs can be layered sequentially by Langmuir-Blodgett (LB) deposition or spontaneously formed from vesicles of lipids in aqueous media [50, 63]. LB deposition can be controlled easily, but it is laborious and difficult to produce in bulk. Vesicle deposition has become popular in the formation of SPBs. The vesicle deposition method provides users with the ability for spatially control the formation of lamellar domains [64]. The behavior of lipid vesicles and the theoretical formation of SPBs were pioneered by McConnell's group in the early 1970's [65]. Many groups utilized native PC lipid molecules and formed SPBs from vesicle solutions to study the

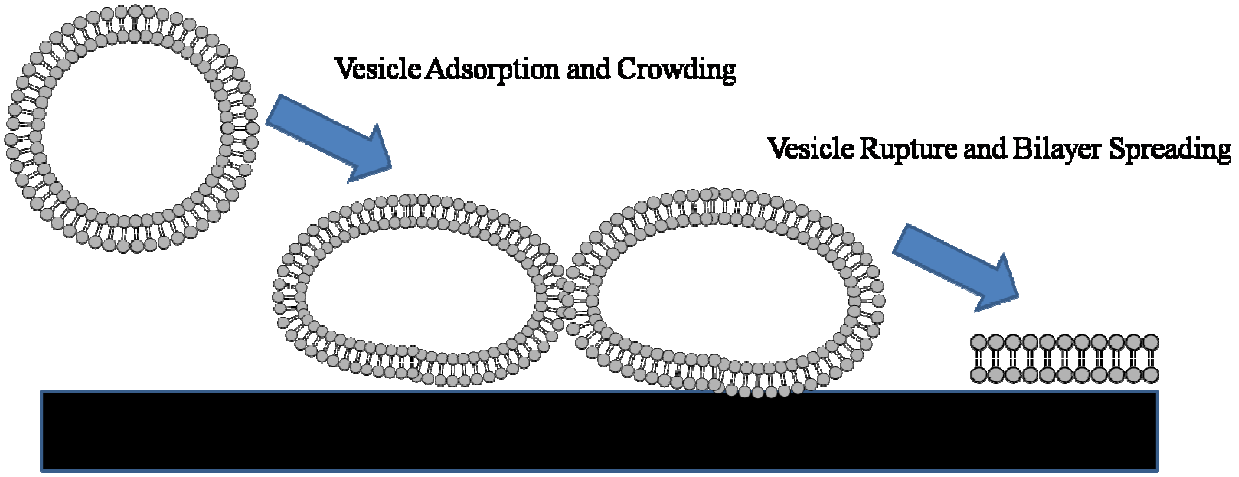


Figure 8. General diagram of vesicle adsorption and spreading onto substrates. The sections of the phospholipid vesicles in contact with the surface have a strong interaction. This interaction causes the vesicles to rupture and begin to spread across the substrate forming a uniform bilayer.

interaction with proteins and cells [49, 51]. Quartz crystal microbalance (QCM), atomic force microscopy (AFM), surface plasmon resonance spectroscopy (SPR) and fluorescence microscopy (FM) were utilized to determine highly quantitative experimental understanding of vesicle formation and interaction with proteins and cells [56, 66-69].

Large numbers of proteins and cells were analyzed with similar results: the large hydration shell formed around the tightly packed membranes prevents the irreversible adsorption of proteins which then prevents cellular attachment [46, 51, 56, 70-73]. The main problem with these systems was the innate instability when removed from the aqueous environment. The weak interactions holding the SPBs to the surface break down when the water is removed. Several methods to stabilize the bilayers have been prepared including polymer cushions, polymer tethers, protein tethering, and polymerization of the bilayers [69]. Polymerization of the bilayers has produced the largest amount of success [74].

One of the first uses of PPBs, completed by Chapman's group, was polymerizable PC diacetylene lipids, shown in Fig. 9 [46]. These systems showed promise and were widely utilized, but were plagued with difficult polymerization and issues with practical application of the system. Around the same time O'Brien's group was experimenting with various unique polymerizable lipid molecules [75].

After several years of study and optimization, a quantitative method for synthesizing and generating stable PPBs composed of 1,2-bis[10-(2',4'-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphocholine (bis-SorbPC or BSPC) was completed [55, 75-76]. This system, detailed later, combines the packing density of native structures and the stability of acrylic phospholipid polymers. The current work is a derivative of O'Brien's pioneering efforts.

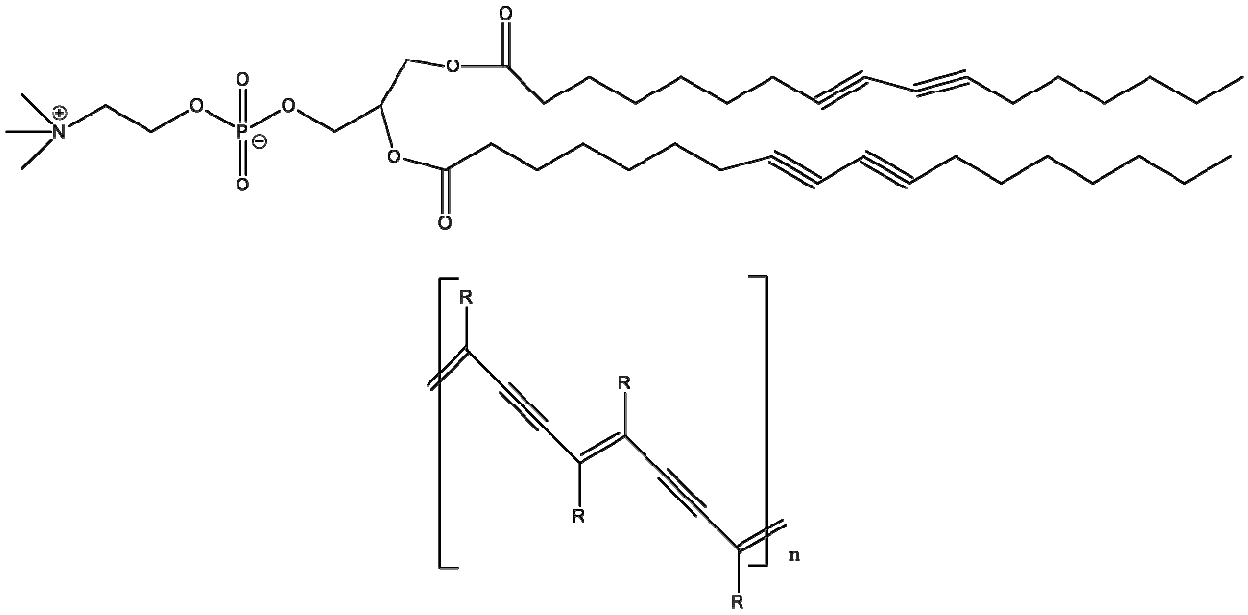


Figure 9. Chemical structure of a diacetylene functionalized phospholipid and the resulting polymerized network structure. The complex packing structure of the alkyl tails, caused by the large diacetylene moieties, disrupted the polymerization efficiency.

1.2 Motivation

Synthetic, biomimetic membranes that are composed of phospholipids seem to be ideal candidates for mediating the host immune response. These natural, amphiphilic molecules self-assemble into a variety of structures depending on concentration, solvent, preparation, and processing [43, 77]. One of the most useful structures is the lamellar bilayer. The major obstacle that must be overcome when using lipid bilayers as material coatings is the intrinsic instability that occurs when they are exposed to air, which causes near quantitative detachment from the substrate. Several methods, mentioned previously, have been developed to modify lipid bilayers in order to stabilize the interface in air. These include, but are not limited to, crosslinking polymerization, adsorption or grafting of hydrophilic polymers, and the addition of bolaamphiphiles. The most effective method of stabilizing these bilayers seems to be crosslinking the hydrophobic region. These PPBs are known to have similar protein resistance to fluid lipid bilayers [57].

The application of a practical, stable and biocompatible non-fouling surface has an extreme relevance with the advance of biomedical devices. Myriads of devices would benefit from mediating the host response. Specifically it would be beneficial in situations where cellular invasions would render the device inactive, such as sensors that dynamically monitor biofeedback. These sensors are typically fabricated out of silicon. Development of a stable, thin coating to prevent protein and cellular fouling, and keep the sensitivity of the sensor intact, would enable long-term application of these devices.

This thesis involves the application of a well studied PPBs system and its interaction with a modeled *in vitro* host response. PPBs were generated on silicon wafers in order to model a planar substrate with relevance in biosensor applications. The stability of the PPBs was tested under physiological conditions and a proof of concept for future functionalization was performed. Cellular adhesion was analyzed along with phenotypical expression on PPBs. Typical image processing was conducted with ImageJ software.

1.3 Thesis Outline

Chapter 2 focuses on quantitatively measuring the physical properties of the PPBs on the silicon supports. Ellipsometry and contact angle measurements are taken and utilized to conclude an understanding of surface chemistry and bilayer uniformity. Fluorescent microscopy is also utilized to garner knowledge of bilayer homogeneity. The stability of the PPBs is tested in simulated physiological conditions. Finally, layer-by-layer (LbL) functionalization of the PPBs was attempted as a proof of concept for future functionalization of the system.

Chapter 3 discusses *in vitro* analysis of murine derived macrophages and fibroblasts, key cells in the host response, to determine the interactions with PPBs. The window of study for these cells is up to 48 hours or in the early attachment phase. Fluorescent and scanning electron microscopy (SEM) were utilized to elucidate the morphological responses of the given cells. ImageJ analysis was performed to quantitatively analyze the morphological differences between cells on PPBs and bare silicon controls.

CHAPTER 2

2.1 Generation and Characterization of PPBs of bis-SorbPC

2.1.1 Introduction

As mentioned above PPBs were optimized in Chapman and O'Brien's early work with alkene functionalized phospholipid molecules [78-80]. Motivated by the inherent problems in alkyne phospholipid molecules, O'Brien analyzed and studied the polymerization mechanisms of acryloyl, methacryloyl, sorbyl and dienoyl functionalized phospholipid molecules [55]. The benefits of these systems were that the polymerization was not nearly as sensitive and could be completed by thermal, redox, light, or γ initiation [55]. Several studies were conducted to elucidate the polymerization mechanism, and optimize the stability, degree of conversion, and polymerized structure [55, 75]. Several factors, including polymerizable group placement, reactivity, spacer length, and initiator, determined the outcome of the polymerizations [55, 79]. Early work, completed by O'Brien and co-workers, utilized this knowledge to generate a targeted drug delivery liposome [76]. Mixed liposomes were prepared with polymerizable and non-polymerizable lipid molecules. They determined that during polymerization a phase separation of the non-polymerizable lipids occurred, causing destabilization of the liposome wall [76]. The controllable destabilization would lead to leakage of the contained therapeutic. These works lead others in O'Brien's research team to investigate SPBs of alkene functionalized phospholipids [56].

Similar optimizations were performed by Ross et al. in determining the most effective molecules for stable PPBs [52, 56-57]. It was determined that PPBs composed of bis-SorbPC

provided uniform bilayers stable against solvents and air, and resistant to bovine serum albumin adsorption, even after drying and subsequent rehydration [52, 57]. A significant finding of this research was that the polymerization technique of bis-SorbPC greatly affects is stability as a PPB. The authors determined that UV initiation generated far less stable bilayers than redox initiated samples [52]. The UV initiated samples generated PPBs that had low molecular weight oligimers rather than fully crosslinked networks seen by samples initiated with a redox pair [52]. Since the early work conducted on this system, bis-SorbPC PPBs have been applied to reusable capillary coatings for protein separation columns, drug delivery vesicles, high efficiency nano-imaging, and high throughput chemical sensing [81]. However, this system has yet to be tested in the arena of controlling the interface between synthetic biomaterials and the host immune system.

PPBs composed of bis-SorbPC have several benefits over other non-fouling surfaces, such as SAMs and phospholipid polymers. SAMs have been thoroughly tested *in vitro* with both protein and cell adsorption, but recently Jones et al. found that these materials may not be suitable to model the host response for longer time frames [82]. It was reported that after only two hours, large topographical changes developed on various surface terminated SAMs, and this altered their non-fouling properties [82]. Alternatively, phospholipid grafted polymers have shown a great deal of success in limiting cellular and protein adhesion, but require further research in order to improve several properties including the packing density of the phospholipid grafts, mechanical properties, and degradation products. The PPB system utilized in this work should be able to combine the advantages of the excellent packing density of SAMs and inherent stability in air of phospholipid polymers while limiting the disadvantages.

2.2 Methods and Materials

2.2.1 Materials

Bis-SorbPC was prepared by modifying the procedure followed by Lamparski et al [76]. The concentration of the stock solution or HPLC-purified bis-SorbPC was determined on a UV-Vis spectrometer (Biomate 5, Thermospectronic, Madison, WI) versus a methanol blank (data shown in Appendix A). Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine DHPE, Invitrogen, Carlsbad, CA) was dissolved in chloroform to a concentration of 5 mg/mL. Silicon wafers with a 100 nm surface layer of silicon dioxide were purchased from University Wafer (South Boston, MA). All other reagents and solvents were commercially available and used as received. Ultrapure water was obtained from a Millipore (Billerica, MA) Direct-Q system with a measured resistivity of 18.2 MΩ.

2.2.2 Surface treatment

Silicon wafers were cut into 5 mm by 5 mm squares. The wafers were cleaned by sonication in acetone, ethanol, and ultrapure water three times for each. The wafers were dried and stored until future use. Directly prior to vesicle deposition, the wafers were treated with boiling piranha solution (3:1 concentrated sulfuric acid/30% hydrogen peroxide v/v). *Caution: Piranha solution is extremely corrosive and requires delicate care during preparation, handling, and disposal.* The wafers were then rinsed and sonicated in copious amounts of ultrapure water.

2.2.3 Vesicle generation and bilayer formation

Bis-SorbPC was dried from the stock solution of chloroform with a stream of nitrogen. Two sets of samples were prepared (1) 100% bis-SorbPC or (2) bis-SorbPC fluorescently doped

with 1.5 mol% of rhodamine DHPE. The dried lipids were placed in a vacuum desiccator for two hours to remove residual solvent. The lipids were then suspended in ultrapure water at a final concentration of 0.5 mg/mL. The aqueous suspension was vortexed and sonicated to clarity in a bath type sonicator (Branson, Danbury, CT). The temperature of the water bath was maintained above the lipid main phase transition temperature (T_m = 28.8 ^{o}C). Aliquots of the vesicle solution were placed on the dried piranha treated wafers. Care was taken to ensure that the vesicle solution did not flow off of the wafer surface, due to the fact that exposure to air has been shown to destroy non-polymerized lipid bilayers.

Continuous bilayer coverage was attainable after 15 minutes. Bilayer coverage was not affected by allowing longer deposition times. In order to remove excess vesicles while preventing bilayer disruption, the substrates coated with lipids were carefully plunged into a shallow glass dish filled with ultrapure water for polymerization.

2.2.4 Polymerization

Redox-initiated polymerization was conducted in ultrapure water deoxygenated by flowing nitrogen. Potassium persulfate (100 mM, Fisher Scientific, Waltham, MA) and sodium bisulfite (10 mM, Sigma-Aldrich, St. Louis, MO) were utilized as the redox initiation pair. The polymerization was continued for two hours under nitrogen atmosphere. After polymerization the lipid coated wafers were sonicated in individual test tubes with a 1% solution of Triton X-100 (Fisher Scientific, Waltham, MA) surfactant and then ultrapure water. The lipid-coated wafers were dried and stored under a nitrogen purged environment.

2.2.5 Bilayer characterization

To determine bilayer thickness, ellipsometric analysis of the dried, lipid-coated wafers was measured with a L0116S ellipsometer (Gaertner Scientific Corporation, Skokie, IL) using He/Ne laser (632 nm) illumination at a 70^{o} incident angle. The spot size was determined to be 1 mm x 3 mm. Baseline readings were obtained from cleaned, bare silicon wafers. The refractive index of the lipid films was assumed to be 1.46 [52]. LGEMP software (Gaertner Scientific Corporation, Skokie, IL) was used to analyze and calculated the PPB coverage. At least three measurements were taken at different locations on each sample. Samples were analyzed in triplicate.

The hydrophilicity of the bare and lipid coated silicon wafers were measured by static contact angles of ultrapure water applied to dried samples. Visual readings were taken from the Model 190-F1 goniometer (Ramé-Hart, Mountain Lakes, NJ). Due to the small size of the samples, only one spot could be taken per wafer. Therefore, measurements were repeated on at least five individually prepared samples to ensure consistency.

Fluorescent images were captured on a Nikon model E-600 microscope for the rhodamine DHPE-doped bilayers to ensure macroscopic uniformity. These samples were used exclusively for imaging purposes because Ross et al. found that addition of a non-polymerizable phospholipid into the bilayer caused the gradual loss of the molecule [52]. This could potentially expose large defects in the bilayer that would disrupt bilayer continuity and interfere with cell culture studies. Examples of this phenomenon are further explained in Appendix B.

2.2.6 Stability tests

As shown in Fig. 10 the phosphatidyl choline (PC) headgroup of BSPC is attached to the hydrophobic tail by two ester bonds. These bonds can be hydrolyzed in basic or acidic

environments, potentially disrupting the bilayer uniformity. In order to characterize the stability of the PPBs, PPB coated wafers were placed in phosphate buffered saline (PBS) solution (Sigma-Aldrich, St. Louis, MO), pH 7.4, at 37 ^{o}C for up to 21 days. At predetermined time points, the wafers were removed, rinsed with ultrapure water, dried with nitrogen, and subjected to surface characterizations as described above. Samples were analyzed in triplicate.

2.2.7 Layer-by-Layer Deposition on PPBs

Aqueous solutions of poly(acrylic acid) (PAA, Polysciences, Inc., Warrington, PA) and poly(N-vinyl pyrrolidone) (PNVP, Sigma-Aldrich, St. Louis, MO) were dissolved in ultrapure water at a concentration of 1 mg/mL. The solutions were adjusted to pH 3. PPB coated wafers were placed in alternating solutions of PAA and PNVP for 5 minutes followed by 1 minute rinses in ultrapure water (pH 3). Samples were dried and analyzed after deposition of subsequent bilayers by ellipsometry.

2.2.8 Statistical Analysis

Statistical significance was determined by Student's *t*-test. A confidence level of 95% is defined as statistically significant. All values are reported as the mean and standard deviation of the mean.

2.3 Results and Discussion

Ross et al. reported studies to optimize the polymerization conditions to generate highly uniform, stable bilayers of bis-SorbPC [52]. In this work the optimized system was utilized for

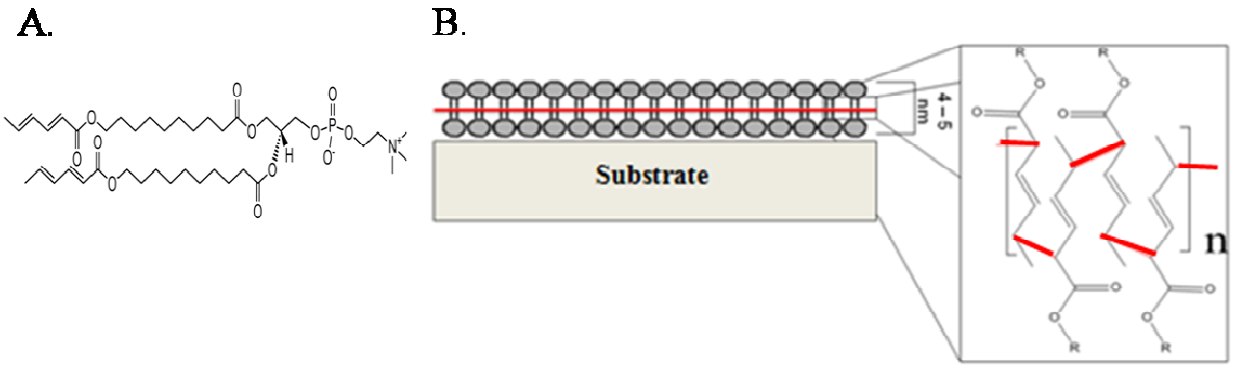


Figure 10. Chemical structure of bis-SorbPC (A), and resulting structure after polymerization (B).

all analysis. Ellipsometric thickness and contact angle measurements for PPBs (Table 3) were used to confirm that proper deposition of the lipids on the wafers had been accomplished. The observed data is within the range of previously published data on redox PPBs of bis-SorbPC and the contact angle indicates that we have achieved a densely packed network of PC headgroups [52]. The hydrophilic nature of this system is crucial for the prevention of protein adsorption, which mediates cellular attachment. Fluorescently doped bilayers confirmed the bilayer uniformity demonstrated by the ellipsometric and contact angle data (Fig. 11). Each PPB coated wafer was sonicated in surfactant and then dried, which indicates the robustness of this system.

As with any coating material, stability is critical to ensure long-term performance. The stability of the PPBs against hydrolysis was measured over a period of 21 days in simulated physiological conditions (Fig. 12). During the incubation period, the PPB thickness declined slightly relative to that of the original dried PPB. The average thickness of the bilayers over the entire range of observation is 38 Å. The decline is likely due to desorption of lower molecular weight sections (i.e. oligomers) of the PPBs during the immersion, drying, and rehydration of the samples at each measurement. Any polymerization is accompanied with incomplete reactions, unintended terminations, and side reactions that could affect function. The redox polymerization has been shown to have especially high degrees of polymerization with bis-SorbPC (Xn ~40 – 600), but some oligomers and some defects could be present [52, 55].

It is worth noting that if the PC headgroup was hydrolyzed, causing the drop in thickness, the contact angle should also decrease. The product of the PC headgroup and glycerol backbone hydrolysis would be carboxylic acid-terminated alkyl chains. The static contact angle for densely packed SAMs of carboxylic acid-terminated alkyl chains on gold is close to 11^{o} [83],

while the contact angle of densely packed PC-terminated alkyl chains on gold is 28^{o} [28]. If the PC headgroup was hydrolyzed, then the contact angle should drop. In this study, a slight increase in water contact angle was observed instead (Fig. 12). This suggests that the PC headgroups in the PPBs were stable against hydrolysis under physiological conditions for at least 21 days. The surface defects in the bilayers, exposing more hydrophobic alkyl chains, would cause the observed increase in the contact angle. The values obtained even after 21 days in PBS agree with previously published data with PPBs of bis-SorbPC. It is assumed that the PPBs maintain uniformity and remained largely undamaged. The cellular interactions with the PPBs after the immersion tests, displayed later, support this assumption.

LbL is a well understood technique for surface modification. There are many systems that could be applied to provide various surface chemistries or functions. The LbL deposition of alternating layers of PAA and PNVP was chosen because of its bonding scheme. This system is bound by hydrogen bonds [84-85]. The carboxylic acid groups on the PAA can donate one or multiple hydrogen atoms to the oxygen's bound to the phosphorus atom in the PC headgroups of the bis-SorbPC, shown in Fig. 13. There could also be electrostatic interactions with the predominantly negatively charged PAA and the zwitterionic headgroups in the PPBs; however this may not be a dominating force. The alternating PNVP acts as the hydrogen acceptor to the PAA basement layer and allows for the continuation of the layers [85]. The thickness for three bilayers was determined for this system on PPBs and was found to be roughly 20 nm per bilayer, shown in Fig. 14. The proof of concept that the PPBs can accept hydrogen bonded LbL

Table 3. Ellipsometric thickness and contact angles for PPBs and bare silicon wafers.

Sample	Average Thickness (Å)	Contact Angle
Bare Silicon[a]	0	$43^{o} \pm 2^{o}$
PPBs	43 ± 4	$26^{o} \pm 3^{o}$

[a] The bare silicon wafers utilized in the contact angle were cleaned with acetone, ethanol and ultrapure water before analysis.

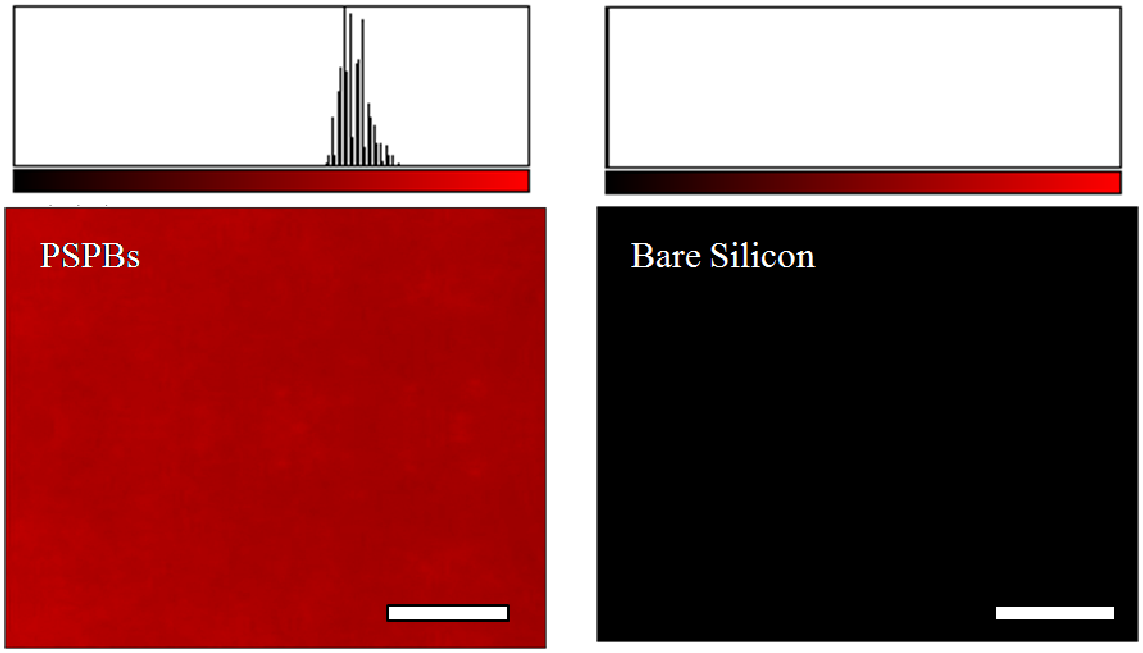


Figure 11. Fluorescent images of PPBs doped with rhodamine DHPE and the bare silicon control. Histograms for each image show the stark contrast of intensity.

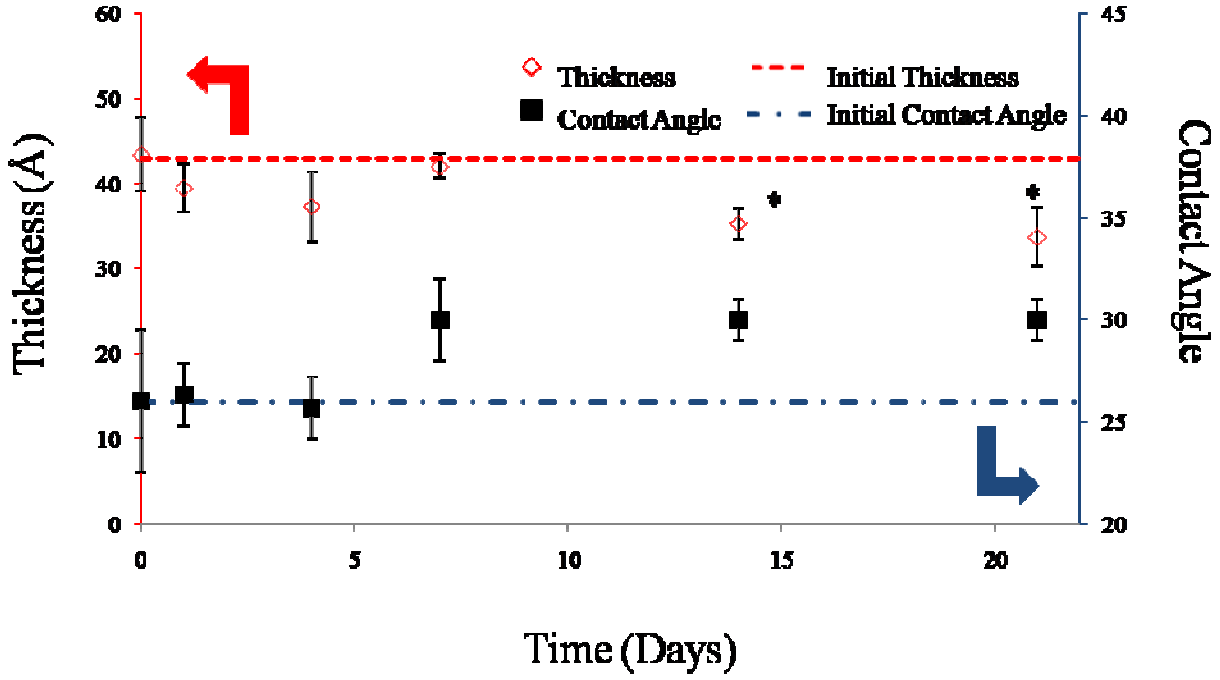


Figure 12. Ellipsometric thickness and contact angle of PPBs immersed for up to 21 days at physiological conditions in PBS.

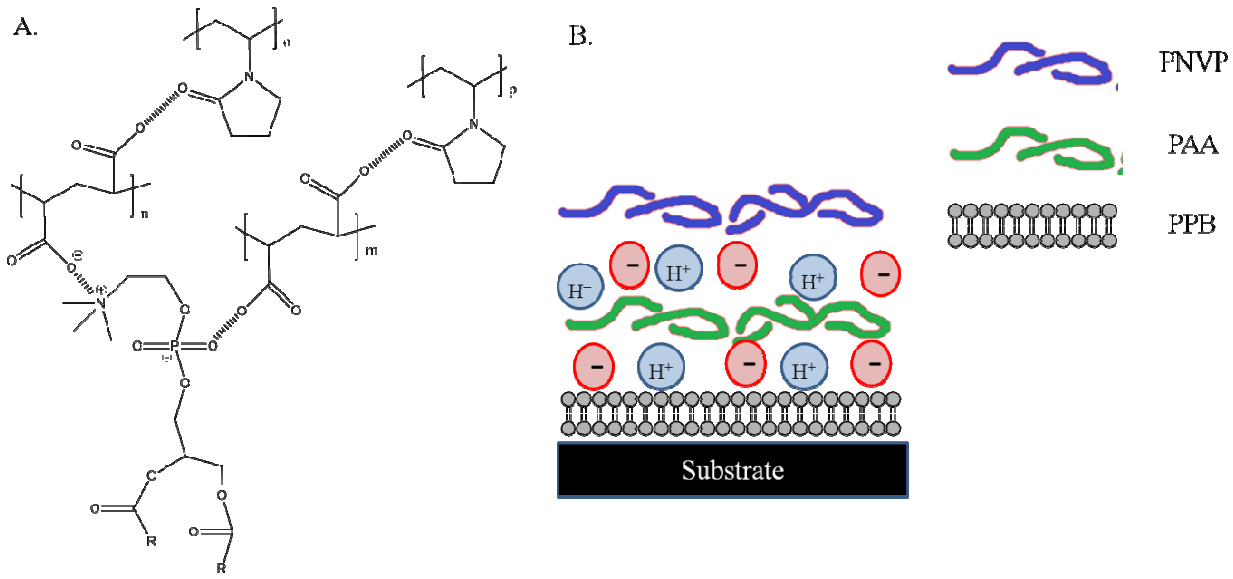


Figure 13. Chemical structure and possible configuration of bound PAA and PNVP layers adsorbed onto PPBs, dashed lines represent hydrogen or ionic bonding (A), generalized diagram of one bilayer of PAA and PNVP bound to the PPBs, H^+ represents hydrogen bonds and (–) represents ionic interactions (B).

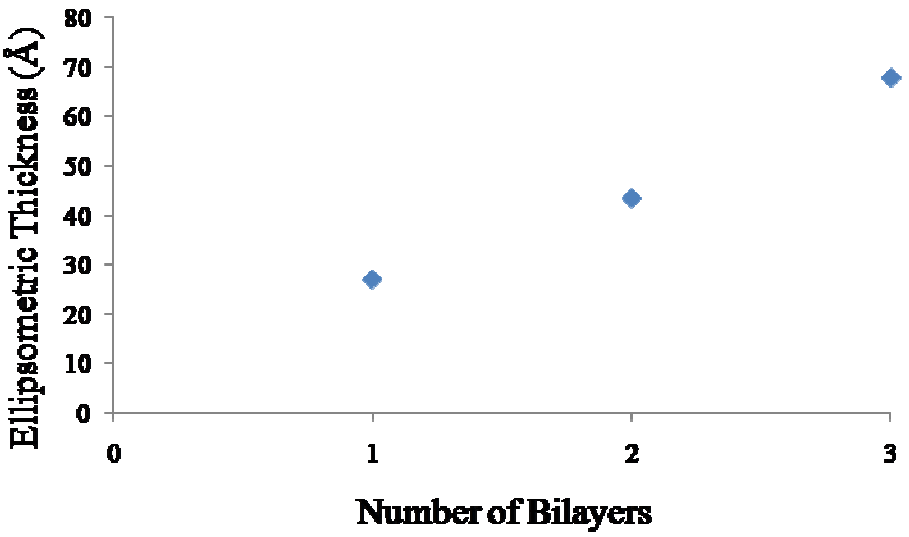


Figure 14. Ellipsometric thickness of PAA and PNVP bilayers deposited onto PPBs.

deposition allows for a myriad of functionalization opportunities. One of which could be combining polymer-drug conjugates to the PNVP monomer in order to release therapeutics [86].

CHAPTER 3

3.1 Cellular Interaction with PPBs of bis-SorbPC

3.1.1 Introduction

As mention above, macrophages and fibroblasts play crucial roles in mediating the host response towards implanted materials. Macrophages are present early in the inflammatory response and continue to proliferate into the later wound healing phases. Activated macrophages (either with pathogens or foreign materials) begin to phenotypically differentiate early in the host response [10]. They undergo a dendritic or spreading morphology to maximize the contact points with the surface [2, 10]. This is their attempt to phagocytose the material. If the macrophages cannot degrade the material they may reach a state of frustrated phagocytosis in which the cells will fuse into FBGCs and begin to engulf the material [2]

Fibroblasts usually accompany macrophages and foreign body giant cells (FBGCs) in the final stages of wound healing [2, 11]. The fibroblasts play a crucial role in angiogenesis and remodeling the extra cellular matrix [1]. Granulation tissue is secreted by the fibroblasts, which can aid the encapsulation of the foreign material [2]. This can lead to device failure or loss of functionality *in vivo* [87]. It would be beneficial to have a biologically inert stable coating that would not only limit cellular attachment, but also prevent additional cellular activation. The goal of this chapter is to address whether PPBs could potentially function to decrease the host response.

This work utilizes immortalized murine derived macrophages and fibroblasts due to their ability to express phenotypic markers similar to that of primary cells. Specifically the RAW

264.7 macrophages show tendencies of dendritic phenotypes when exposed to foreign materials [88-90]. The NIH 3T3 fibroblasts also have a unique phenotypical expression of spindle shaped cell bodies when attached to favorable surfaces. These characteristics allowed for easy identification of the type of attachment. Both cell lines show rounded cell bodies when exposed to unfavorable surfaces. Examples of these phenotypical expressions are displayed in Fig. 15.

3.2 Methods and Materials

3.2.1 Murine macrophage and fibroblast culture

The murine derived macrophage cell line, RAW 264.7, and fibroblast cell line, NIH 3T3, are subclones of lines originally purchased from ATCC (Manassas, VA). Both cell lines were used to model the host response, *in vitro,* to the polymerized lipid bilayers. RAW 264.7 murine macrophages were grown in Dulbecco's Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific Waltham, MA), 1% L-glutamine, and 1% penicillin/streptomycin (P/S). Cells were cultured at 37 ^{o}C, 5% CO_2, and 95% humidity. For passage cells were gently dislodged from the culture dish mechanically and reseeded in a new dish. To study macrophage behavior on the different surfaces, cells were seeded at a density of $2.5x10^4$ cells/well onto the substrates placed in a 48-well plate.

The NIH 3T3 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS, Thermo Scientific, Waltham, MA), 1% L-glutamine, and 1% P/S. This cell line was cultured in a T-25 flask to near confluency before each passage. Standard enzymatic

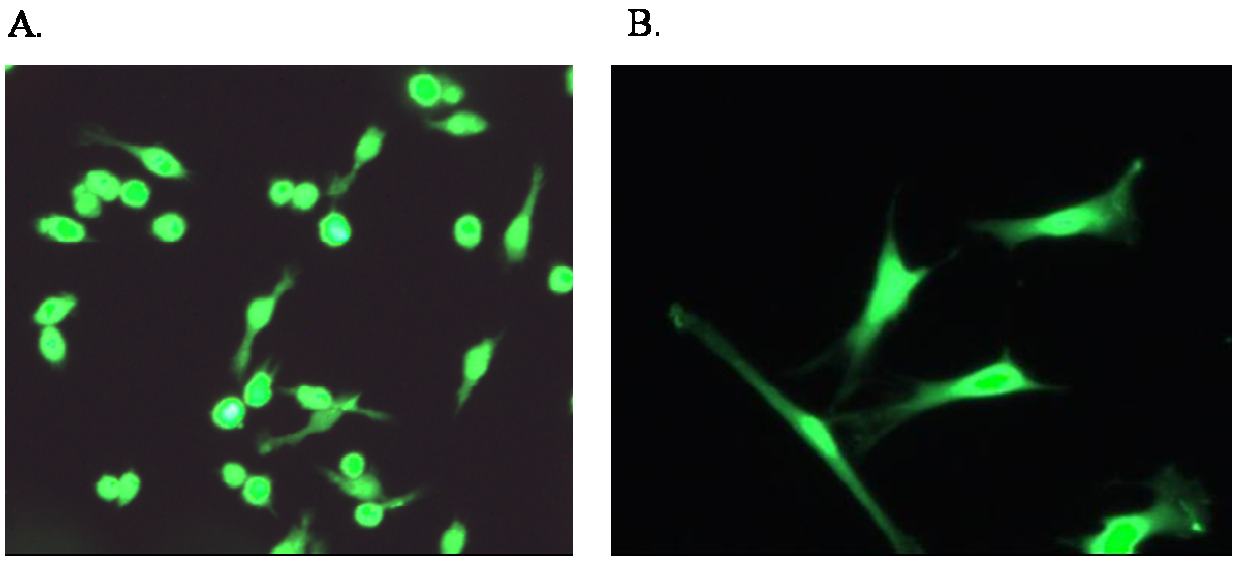


Figure 15. Morphology of RAW 264.7 cells (A) and NIH 3T3 fibroblasts (B).

dissociation with trypsin and replating were used for cell passaging. To study fibroblast behavior, cells were seeded at a density of $5x10^3$ cells/well onto the substrates in a 48-well plate.

For both the macrophages and fibroblasts, cell number and phenotypic response was studied on PPB coated wafers with cleaned, bare silicon wafers used as a positive control for attachment. At various time points a live/dead fluorescent-staining kit (Invitrogen, Carlsbad, CA) was used to enumerate the live and dead cells. Substrates were gently washed with PBS before and after staining.

3.2.2 Cell imaging and analysis

Fluorescent images of the stained cell cultures were captured with a Nikon Eclipse model E-600 inverted microscope with Image Q software. For the macrophage experiments image analysis was conducted with ImageJ, free software distributed by the National Institutes for Health (NIH). For each sample at least 3 representative images were studied. The total number of live cells attached and the number of activated (A) and non-activated (NA) cells was determined. A particle sorting and counting algorithm within the ImageJ program was used to distinguish NA and A. This function allows the user to separate the different cells by their circularity, defined by $4\pi(\text{area}/\text{perimeter}^2)$, and pixel size. A detailed description and examples of this process are described in Appendix C.

Scanning electron microscope (SEM) imaging of the wafers cultured with macrophages was conducted on a LEO 1525 electron microscope. The cells were fixed with a 3% glutaraldehyde solution, counterstained with osmium tetroxide, and sequentially dehydrated with a series of solutions of increasing ethanol concentrations. The samples were dried overnight in a

vacuum desiccator before analysis. Directly prior to analysis the samples were sputtered for 10 seconds with gold. Image acquisition used an accelerating voltage of 5 kV.

3.2.3 Stability Test

In the stability test described in Chapter 2 Section 1.2.6 there were observed changes in the bilayer thickness and contact angle. To ensure the bilayers maintain their non-adhesive properties RAW 264.7 macrophages were cultured for 24 hours onto the samples after removal from the PBS bath. The PPB coated wafers were seeded with RAW 264.7 macrophages at a density of 2.5×10^4 cells/well in a 48-well plate. After 24 hours of culture, the samples were stained with a fluorescent Live/Dead staining kit (Invitrogen, Carlsbad, CA) and imaged for phenotypic state.

3.2.4 Statistical Analysis

Statistical significance was determined by Student's *t*-test. A confidence level of 95% is defined as statistically significant. All values are reported as the mean and standard deviation of the mean.

3.3 Results and Discussion

Protein adsorption is a leading factor behind cellular attachment and behavior on any biomaterial surface [2]. The protein resistant characteristics of lamellar lipid bilayers, phosphorylcholine modified polymers and PPBs of bis-SorbPC have been well studied previously. For example, Ross et al. showed that the surface coverage of nonspecifically adsorbed proteins on a bis-SorbPC PPB is about 1% of a monolayer [57]. It was hypothesized that proteins are adsorbed to defects in the PPB, not the PPB themselves. Knowing that protein

adsorption to PPBs is minimal, this study uses attachment and phenotypical expression of cells to test whether PPB coating can limit the attachment of host response cells.

RAW 264.7 cells were cultured onto PPB coated or bare silicon wafers. As shown in Fig. 16 A, the total number of cells on the PPBs at each time point was significantly below that of the bare silicon control. Dead cells were negligible on each substrate. Representative images from the 24-hour culture (Fig. 16 C) highlight the dramatic differences between the two conditions. Of the cells that were attached to the PPBs, very few displayed the dendritic morphology compared to the control samples. Some of the attached cells on the PPB-coated wafers aggregated together, tethering themselves to other cells, before eventually detaching from the substrate. This aggregation and detachment phenomenon correlates well with previously published data on RAW 264.7 macrophage attachment to phospholipid-based substrates and is representative of non-adhesive surfaces [54]. If the cells cannot find suitable, strong attachment areas they will simply detach from the surface.

To quantify the phenotypic differences between PPB and silicon attached cells, the number of activated and non-activated cells were enumerated using ImageJ analysis (Fig. 16 B). The number of activated cells was significantly less ($p < 0.05$) than that of the bare silicon at each time point. The small number of cells adhering to the PPB surface could be attaching to defects in the PPB coating. We hypothesize that these defects are relatively small in number and spaced far enough apart that the cells that do adhere cannot spread onto the surface. The remaining cells are in fact just clinging to a very small number of contact points. High magnification SEM images of cells on both PPBs and bare silicon wafers, Fig. 17, show that the macrophages that attach to the bare silicon are well attached and spread. Each of the cells has a

large number of filopodia extending in all directions (Fig 17 A). Observed at higher magnification, Fig. 17 B-C, each filopodia has a large number of contact points with the surface. As for the adherent cells on the PPBs, Fig. 17 D-F, there are two aspects that are of particular interest: (1) the number of contact points for the adherent cells is less than those on the control and (2) there is a heterogeneous population of adherent cells.

The limited number of attachment points that PPB-adherent cells correlates with the hypothesis that the cells are only able to attach to defects in the bilayer. Their rounded phenotype suggests their inability to find sufficient attachment sites to support cell spreading. The two main populations of PPB-adherent cells can be distinguished into groups consisting mainly of individual cells and small aggregates of several dozen cells loosely bound to adherent cells. This heterogeneous phenomenon is transient and diminishes after 48 hours. The hypothesis can be made that most cells are unable to find defects on the surface of the PPB coated wafers and the aggregates are attaching to the cells that have found these defects. However, this "self" adhesion is not strong. After a certain time the aggregated cells will detach from the PPBs, leaving only the cells that have a strong attachment on the surface.

The NIH3T3 fibroblasts showed a similar behavior with attachment and phenotypic differences on PPBs. The number of fibroblasts that attached to the PPB coated wafers as opposed to the bare silicon controls was significant ($p < 0.05$) (Fig. 18 A). Also, the fibroblasts undergo a phenotypic change after attaching to the PPBs. The morphology of the fibroblasts that adhere after 24 hours, e.g. rounded, smaller cell bodies, is very different from the bare silicon controls, e.g. spindle shaped, elongated cell bodies (Fig. 18 B). Once again if the cells can find

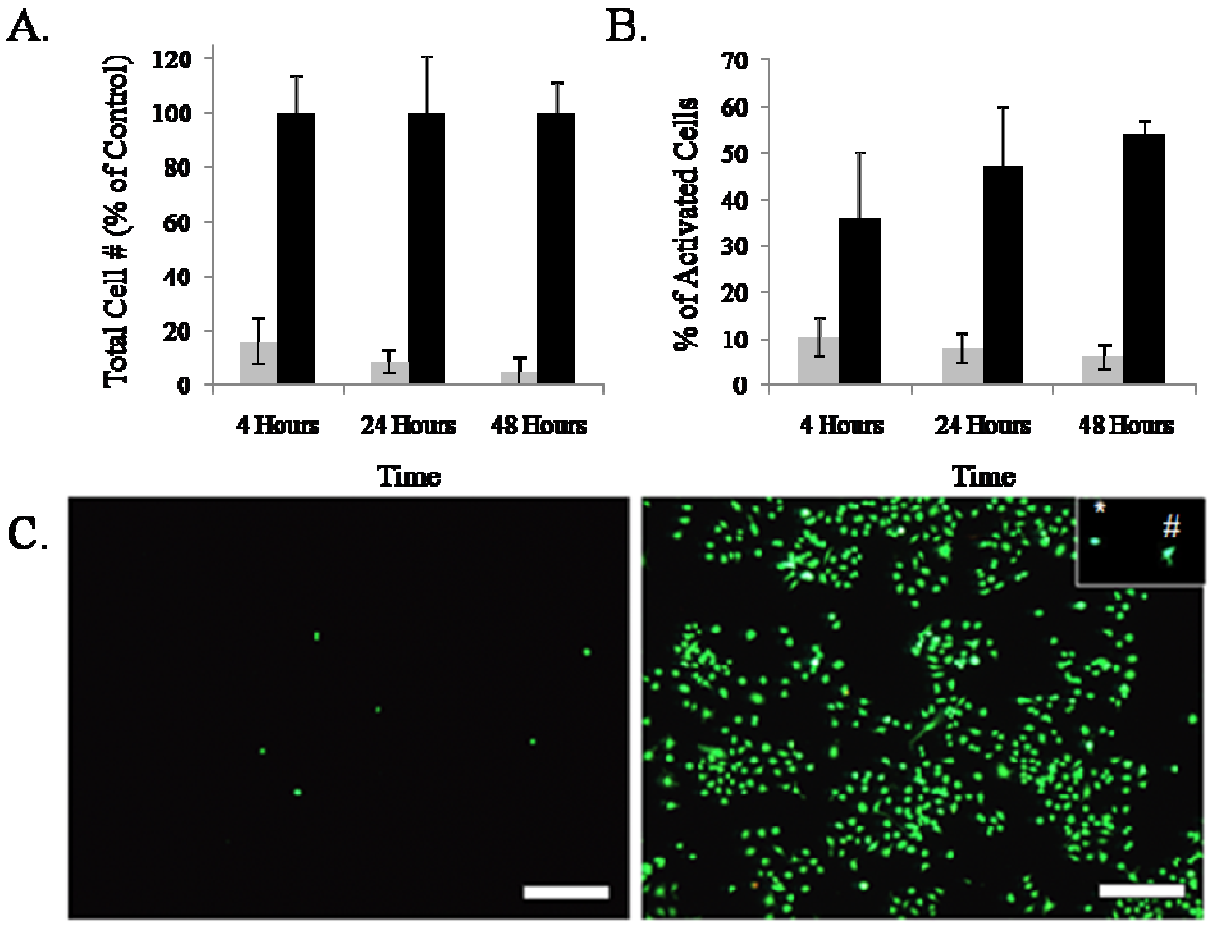


Figure 16. RAW 264.7 cell culture on PPBs. Total cell number on PPBs (grey) with respect to the bare silicon control (black) (A), percentage of cells expressing the spread phenotype on PPBs (grey) and bare silicon (black) (B), representative images showing the disparity between cell populations on PPBs (left) and bare silicon wafers (right) (C). Inset in upper left displays representative quiescent (*) and activated (#) cells. Scale bar is 100 µm.

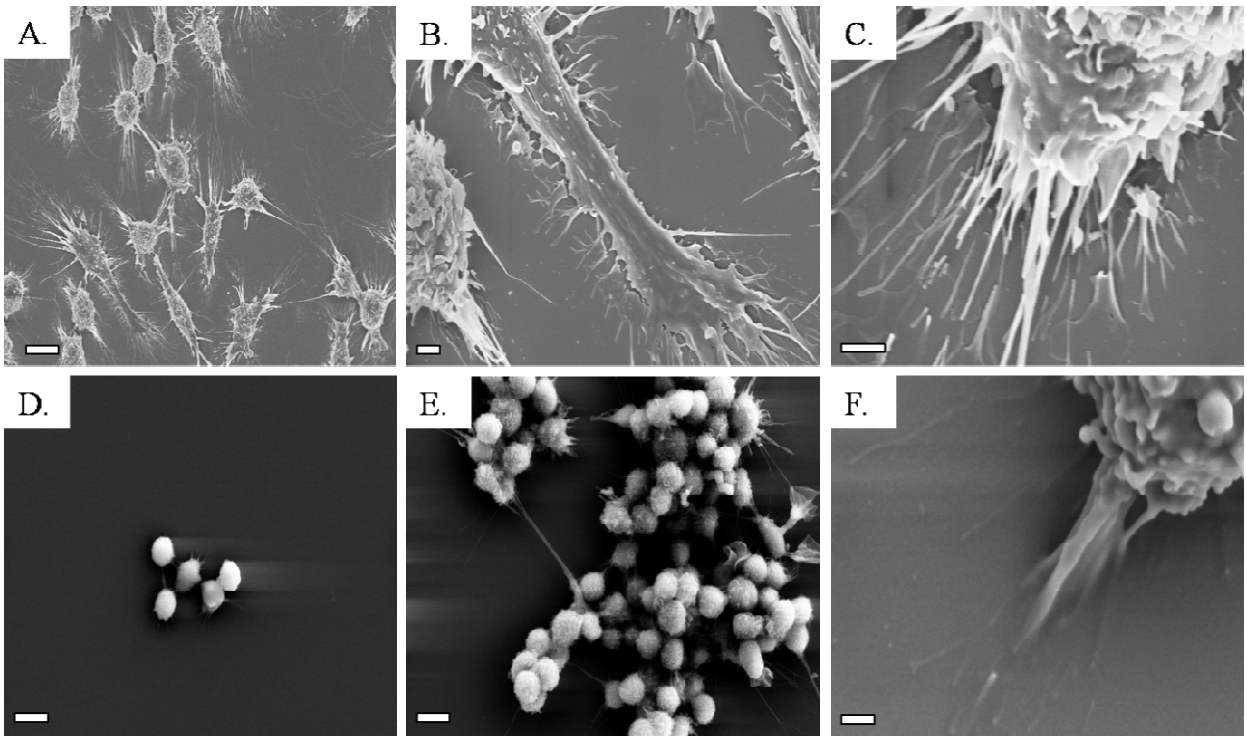


Figure 17. SEM images of RAW 264.7 macrophages on bare silicon (A-C) and PPB coated (D-F) wafers. Scale bar is 10 µm (A-B, D-E) and 1 µm (C, F).

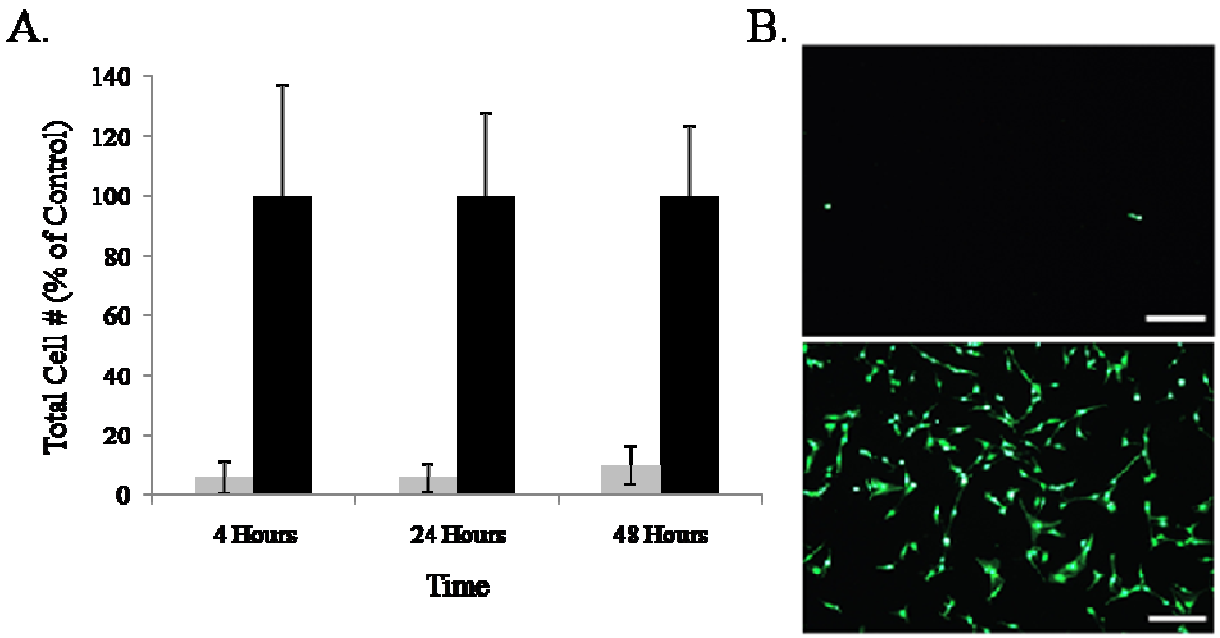


Figure 18. NIH 3T3 fibroblasts cultured onto PPB coated or bare silicon wafers. Total number of cells on PPBs (grey) with respect to the bare silicon controls (black) (A), representative images displaying the differences between PPB coated wafers (top) and bare silicon wafers (bottom) (B). Scale bar is 100 µm.

a defect in the PPB coating, then they will attach. However, because of the limited number of defects in the PPB coating they will not produce the fully spread morphology as seen on the controls.

Both cell lines exhibit similar behavior of limited attachment and altered phenotypic expression. Once the number of cells reaches a steady-state relative to the number of defects, the other cells that cannot attach to the defects detach completely. Those cells that remain are kept in a minimally active state, compared to cells on uncoated substrates. The PPB coatings prevent the initial cellular adhesion after implantation and the few cells that are attached to defects are not fully activated. This limited activation could prevent the eventual encapsulation that is typical of the host response towards biomaterials.

As mentioned above, the PPBs immersed in PBS, at physiological conditions, showed slight changes in bilayer properties. The analysis of the retention of the non-adhesive properties of the PPBs after the stability test was completed with a 24 hour culture of RAW 264.7 macrophages. The PPB coated wafers maintained their non-adherent properties irrespective of the timeframe of immersion in PBS. The fluorescent images shown in Fig. 19 A demonstrate the marked difference between PPB samples and bare silicon controls even after 21 days of immersion in PBS. Both the number of cells and the activation states are similar to that of the initial PPBs (Fig. 19 B-C). The change in bilayer properties did not alter the adhesive properties of the system. This provides evidence that the PPB system can withstand physiological conditions, and maintain their properties, for at least 21 days.

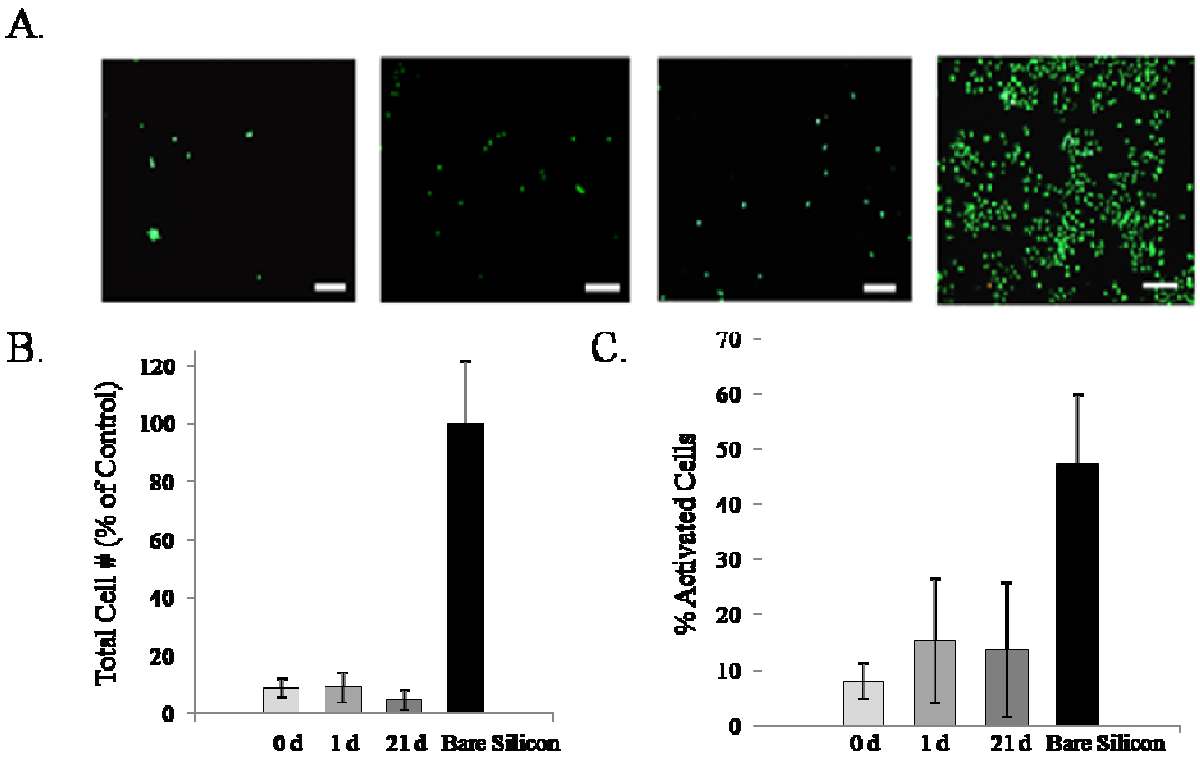


Figure 19. RAW 264.7 cultures on PPB coated wafers after keeping the substrates in PBS for up to 21 days. The cells were cultured for 24 hours after the substrates were dried and analyzed. Representative fluorescent images for 0, 1, and 21 days in PBS and bare silicon wafers (A), total number of cells with respect to the bare silicon control (B), percentage of activated cells (C). Scale bar is 100 µm.

CHAPTER 4

4.1 Conclusions

The overall goals of this thesis were to generate, analyze, and test PPBs as a potential mediator for host material interactions. The PPB coated wafers utilized in this work were found to be macroscopically uniform and within the ranges of other well-developed bis-SorbPC based PPBs. This coating maintained its physical properties even after prolonged exposure to physiological conditions. Furthermore, the ability to retain strongly bound LbL polymeric systems allows for a wide range of future functionalization. The *in vitro* studies showed that PPBs of bis-SorbPC coated on silicon substrates can limit attachment of cells involved in the host response to foreign material. Two cell lines, which represent important populations in the host response to biomaterials, verified that PPBs could prevent initial adhesion and phenotypic changes in representative cells of the acute inflammatory and late-stage granulation responses. The intrinsic non-adhesive property of this system demonstrates indications that PPBs are an ideal platform for biomaterial coatings. The PPBs analyzed in this work warrant further research in both *in vitro* and *in vivo* to examine the effectiveness of PPB coatings on mediating inflammatory and wound healing responses around biomaterial implants. The system studied in this work draws from the pioneering effort of O'Brien's and Saavedra's group. The molecule bis-SorbPC has already been proven to be highly adept in forming PPBs thanks in part to the location and reactivity of its bis-sorbyl moiety. This current adaptation of the bis-SorbPC system shows promise as a mediator of the host response. However, research is still warranted to elucidate cellular attachment and activation states. AFM has been proven to be significant in determining the presence of defects in PPBs, and this technique could be applied to better understand the attachment mechanisms seen in this work. Another invaluable tool to better

determine the exact state of the attached cells is immuno-histochemistry. The integrins expressed by the attached cells could be analyzed to provide intricate knowledge of the activation states. These tests will certainly be conducted in the future along with a number of possible research paths.

4.2 Future Works

A large amount of research has been applied to non-fouling systems and their applications in the field of biomaterials. However, no one system provides the ultimate goal of a true mediator of host material interactions. The PPBs analyzed in this thesis shows promise as a non-fouling surface, but the application of this system may require further functionalization. The research that can be completed with this system focuses on the ability to customize and functionalize the PPB. There is a wide range of research paths to take, such as (1) addition of reactive functional groups in the PPB (2) combining multiple polymerizable phospholipids and transmembrane proteins can be applied to better mimic cell membranes (3) LbL adsorption of polymers containing therapeutics could be utilized to target un-wanted damages from the host response, and (4) conjugation of bioactive molecules to target cellular attachment.

Chemical modification of the polar headgroups in polymerizable phospholipid molecules can be utilized to present functional groups towards at the surface of the PPBs by following a procedure developed by Bae et al. [91]. This system applies a much simpler, three step, method of synthesizing bis-sorbyl functionalized phospholipids. The polar headgroup could be altered from PC to PS, PE, or even non-phosphate based reactive groups. These groups could be utilized to further functionalize the bilayers and make them more biologically active. Similar work has been completed recently with collagen functionalized planar supported phospholipid

membranes [92]. With simple chemistry any phospholipid can be functionalized into the polymerizable derivative. This will allow for the creation of much more specific biomimetic cell membranes. Researchers have also recently incorporated functional transmembrane proteins into PPBs [81]. This can be coupled with the synthetic bilayers to generate highly accurate synthetic membranes. These biomimetic membranes can have applications in tissue engineering, stealth drug delivery, artificial cells, non-fouling coatings, and biochemistry.

The ability of the PPBs to attach polymeric materials with the LbL technique demonstrated in this work opens the door for a large number of possible applications. Polymeric materials or natural polysaccharides could be layered onto the PPBs and provide therapeutic release upon physiological stimulation [86]. This could generate reactive coatings for biosensors that could better target the adverse host response and lead to longer life times of the implants.

Finally, conjugation of bioactive molecules, like binding domains of peptide sequences, can be tethered to the PPBs by either covalent linking or adsorption of a functionalized polymer. This could allow bioengineers to specifically target cells or proteins of interest and prevent non-specific attachment. Overall, this work provides a glimpse at the immense possibilities of the PPBs systems.

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APPENDIX

Appendix A

UV-Vis Determination of bis-SorbPC Concentration

Bis-SorbPC is generally kept in chloroform for storage purposes. When generating a PPB the exact concentration of the stock solution of phospholipids is paramount. UV-Vis is an excellent tool to ensure the exact concentration of stored solutions. The molar absorptivity of bis-SorbPC is 47,100 M^{-1} cm^{-1} at 258 nm. Utilizing Beer-Lamberts law, eq. 2, the concentration can be easily determined.

$$A = \varepsilon bc$$

where A is the absorbance, ε is the molar absorption, b is the path length and c is the concentration.

Methanol is the solvent of choice when performing the spectroscopy for this system, as it does not lay an overlap absorption in the area of interest. Generally 2 µl of solution placed in 1 ml of methanol with a 1 ml methanol blank provides a useful spectra. An example of the UV-Vis spectra of bis-SorbPC is shown in Fig. 20.

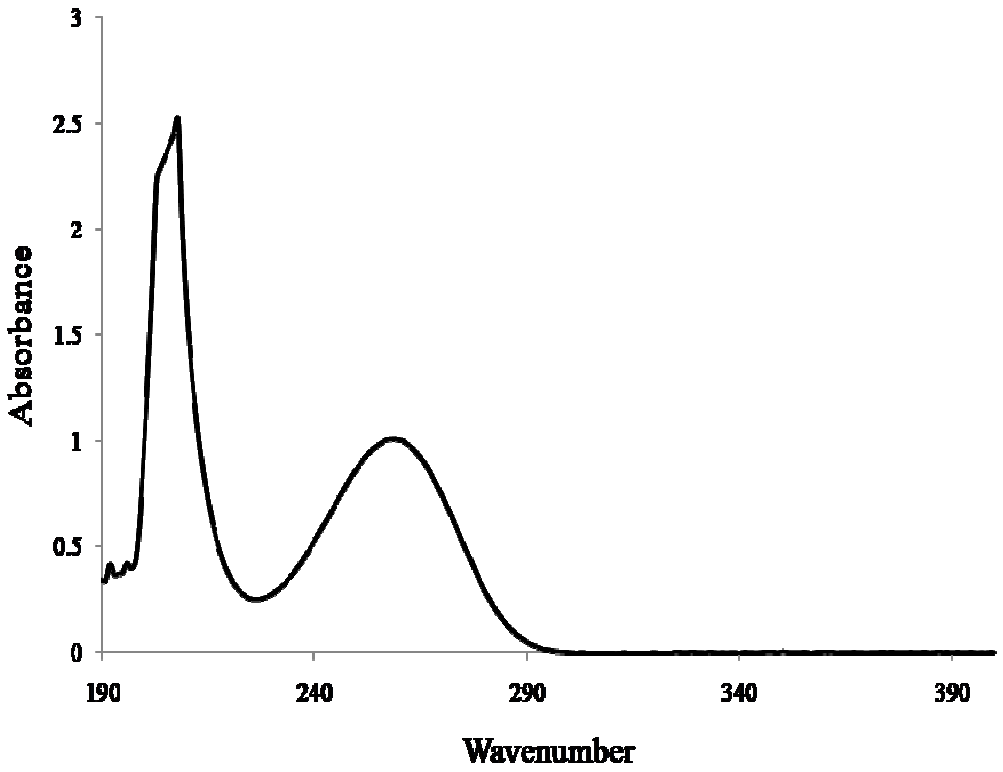


Figure 20. UV-Vis absorption spectra of bis-SorbPC.

Appendix B

Cell Culture on Rhodamine-DHPE doped PPBs

Preliminary studies were completed with rhodamine-DHPE doped PPBs in order to better visualize the cell-surface interactions. These PPBs were generated as mentioned in Chapter 2 section 1.2.3. The PPBs were dried, cleaned with a surfactant solution and analyzed. The ellipsometric thickness and contact angles were similar to those shown in Table 4. Fluorescent images of the PPBs seemed to show macroscopic uniformity. RAW 264.7 macrophages were cultured onto these substrates along with bare silicon controls for 24 hours. The cells were stained, protocol mentioned previously, and imaged. The resulting images, shown in Fig. 21 A-B, were analyzed for total cell number and cells that exhibited activated phenotypical expressions, displayed in Fig. 21 C-D.

The total cell number on the PPB was nearly identical to that of the bare silicon control. However, the cells had greatly different phenotypical expressions. As mentioned earlier, adding in non-polymerizable phospholipids to a PPB can result in desorption of the non-covalently linked molecules. This could expose more defects in the bilayers than are present in the non-doped PPBs. The defect density would be greater, but it appears as though the PPBs prevent the cells that do attach from attaining the spread phenotype seen on the bare silicon controls. The assumption can be made that PPBs doped with any non-polymerizable phospholipid will not provide the same function as a fully crosslinked PPBs.

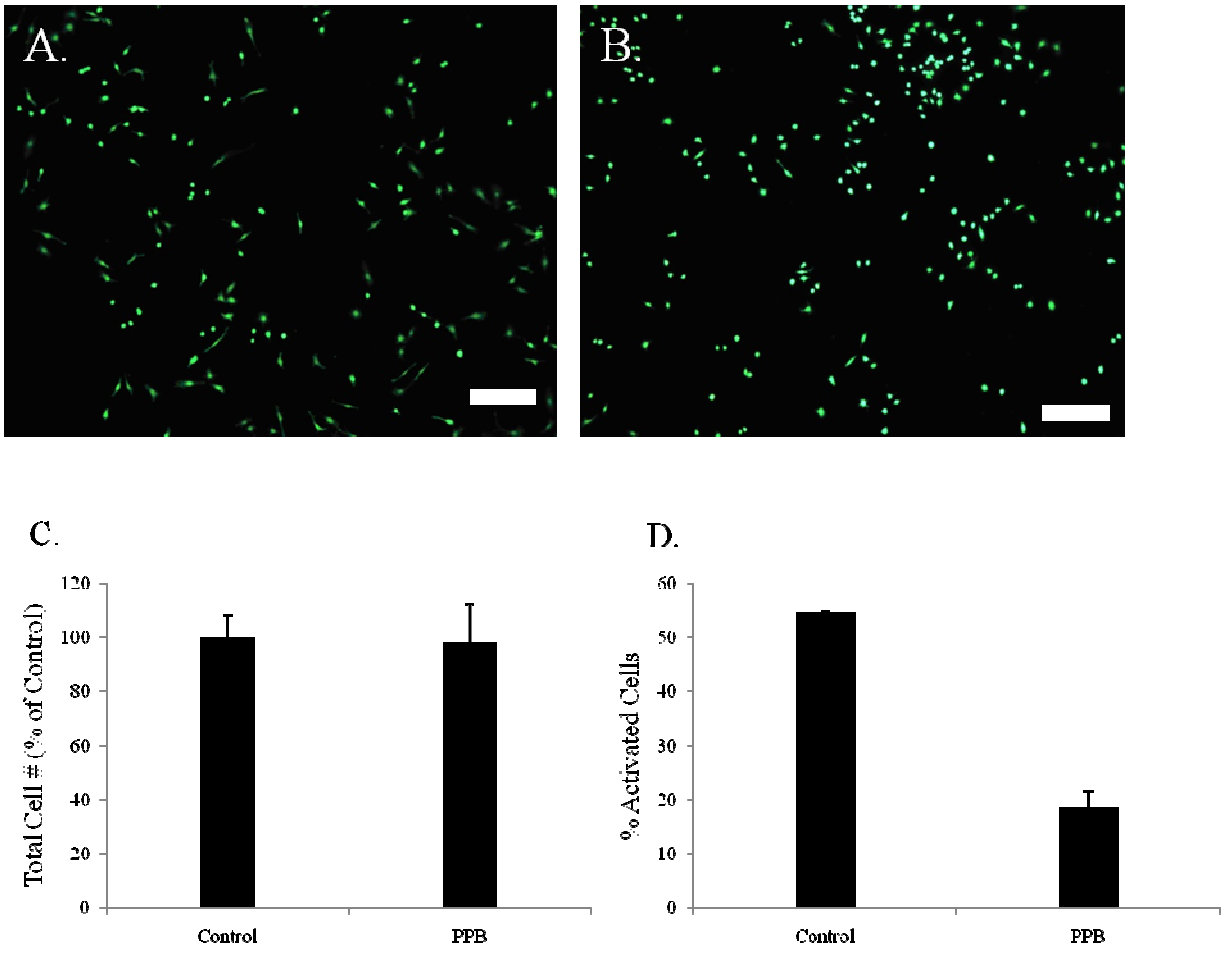


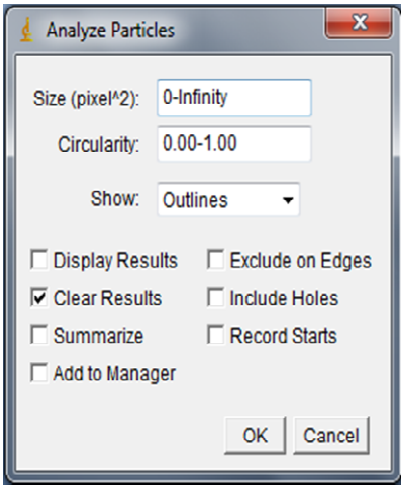
Figure 21. Representative fluorescent images of RAW 264.7 macrophage culture on bare silicon (A) and PPBs doped with 1.5 mol% rhodamine-DHPE (B). Scale bar is 100 µm. Total number of cells as a percentage of control (C) and percentage of cells expressing the activated phenotype (D) for bare silicon and PPB doped with 1.5 mol% rhodamine-DHPE coated wafers.

Appendix C

ImageJ Analysis

All image analysis was performed with the analyze particle algorithm in ImageJ. The images are originally in the RGB format and must be converted to binary before they can be processed. This conversion can be completed in ImageJ. The analyze particle algorithm triggers a GUI interface that is shown in Fig. 22 A. This interface asks to input the size, in units of square pixels and ranging from zero to infinity, and the circularity, ranging from, zero to one. The size range used for the analysis in this thesis was 150-1000. The lower limit for the size was to eliminate counting of noise created when transitioning the image from RGB to binary. The circularity was from 0.8-1, almost perfectly round, for non-activated cells and 0-1 to include all cells in the image. The number of activated cells was determined by subtracting the total number from that of the nearly perfectly rounded cells. Clusters of cells, which could not be individually accounted for with the sorting algorithm, were counted manually. Fig. 22 B displays a representative image of how this technique was applied.

A.



B.

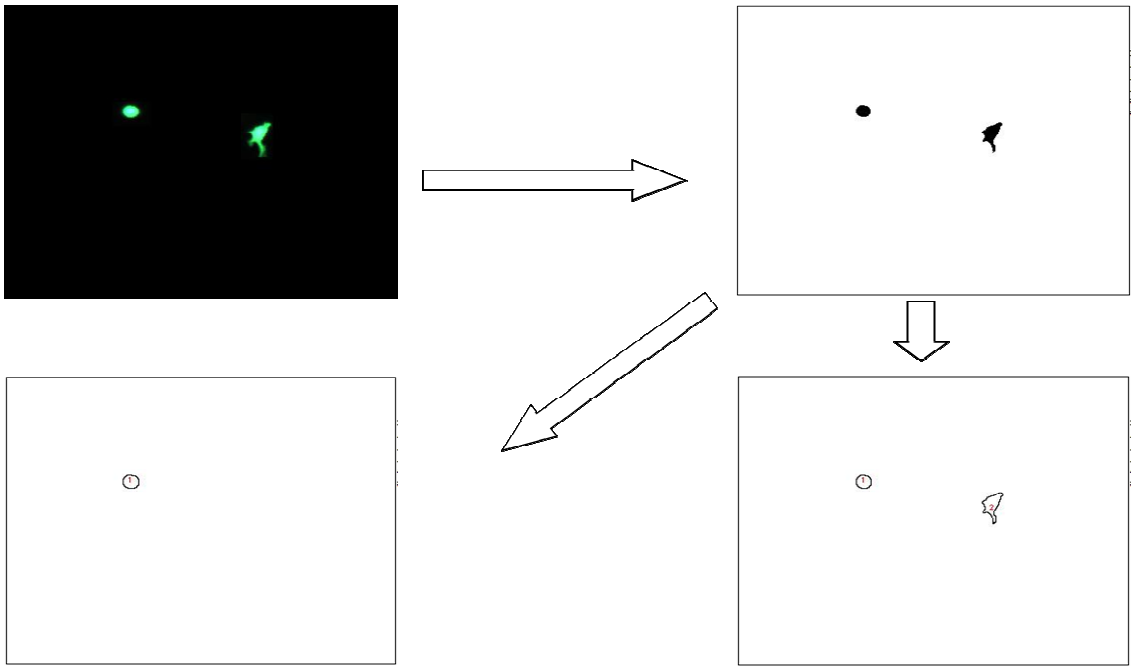


Figure 22. The graphical user interface (GUI) prompted when utilizing the analyze particle function in ImageJ (A), representative image set depicting the image analysis performed in this thesis (B).

VITA

Jonathan Michael Page was born on March 26th, 1986, outside of Nashville, TN. He received a Bachelor's degree in Biomedical Engineering with a minor in Material Science and Engineering from the University of Tennessee, Knoxville in 2008. He then enrolled in the Polymer Engineering Masters program at the same institution in the fall of 2008. After the completion of his Masters Jonathan plans on attending Vanderbilt University in Nashville, TN to attain his doctoral degree in Chemical and Biomolecular Engineering.