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***In vitro* assessment of macrophage attachment and phenotype on polymerized phospholipid bilayers**

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Abstract

Phosphatidyl choline (PC) based materials have been found to be resistant to non-specific protein adhesion *in vitro*. In this study, a PC based planar supported phospholipid bilayer composed of 1,2-bis[10-(2',4'-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphocholine (bis-SorbPC or BSPPC) was generated on piranha treated silicon wafers by vesicle deposition. The bilayer was polymerized with redox initiation forming a stable 4 nm thick coating. Polymerized lipid bilayers (PLBs) were characterized and tested for uniformity, with ellipsometry and contact angle. Cellular adhesion and morphological changes in RAW 264.7 macrophages were investigated *in vitro* on PLBs and compared to bare silicon controls. Fluorescent and scanning electron microscopy were used to observe changes in cellular morphology. The PLBs showed much lower cellular adhesion than bare silicon controls. Of the cells that attached to the PLBs, a very low percentage showed the same morphological expressions seen on the controls. It is hypothesized that proteins adsorb to the defects in the PLBs, caused by incomplete polymerization, and this mediates the observed minimal cellular attachment and morphological changes.

Keywords

Polymerized lipid bilayers; bis-SorbPC; host response; macrophage; RAW 264.7

INTRODUCTION

In recent decades, biomimetic surfaces designed to resist protein adsorption and cellular adhesion have been prepared and characterized. In particular, phospholipids, which comprise a large majority of mammalian cell membranes, have been widely utilized for this purpose.¹⁻⁵ The choice of lipid molecule utilized is of great importance. The zwitterionic phosphatidyl choline (PC) based lipids have proven to be highly protein resistant.^{4,6} Synthetic, biomimetic membranes that are composed of PC based phospholipids are ideal candidates for mediating the host response. These amphiphilic molecules self-assemble into a variety of structures depending on concentration, solvent, preparation, and processing.⁷ One of the most widely analyzed and utilized structure is the lamellar bilayer.⁸⁻⁹ The major

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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.²

Several methods 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.¹⁰⁻¹¹ The most effective method of stabilizing these bilayers is crosslinking the hydrophobic region.² These polymerized lipid bilayers (PLBs) are known to have similar protein resistance to fluid lipid bilayers.³ A specific molecule that has been proven to readily form stabilized lipid bilayers after crosslinking is 1,2-bis[10-(2',4'-hexadienoxy)decanoyl]-*sn*-glycero-3-phosphocholine (bis-SorbPC or BSPC).² Planar supported polymerized lipid bilayers of bis-SorbPC have been shown to be highly uniform and stable against solvents and air.²⁻³ This system has been applied to reusable capillary coatings for protein separation columns, drug delivery vesicles, high efficiency nano-imaging, and high throughput chemical sensing.¹² However, this system has yet to be tested in the arena of controlling the interface between synthetic biomaterials and the host system.

PLBs composed of BSPC have several benefits over traditional “non-fouling” surfaces and other PLB systems. Ross et al. found that PLBs composed of BSPC prevented protein fouling much better than other PLB systems, such as diacetylene functionalized lipids.³ Self-assembled monolayers (SAMs) have been thoroughly tested *in vitro* with protein adsorption, but recently Jones et al. found that these materials may not be suitable to model the host response for longer time frames.¹³ It was reported that after only two hours under physiological conditions, large topographical changes developed on various surface terminated SAMs, and this altered their “non-fouling” properties.¹³ Phospholipid grafted polymers (PGPs), such as 2-methacryloyloxyethyl phosphorylcholine (MPC), 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.⁴⁻⁶ The BSPC system offers the opportunity to combine the advantages of the excellent packing density of SAMs and inherent stability in air of PGPs while limiting the disadvantages.

In this study the interactions of murine derived macrophages with planar supported PLBs composed of BSPC on silicon substrates were investigated. Macrophages were chosen due to their relevance in the inflammatory phase of the host response. This is the first report showing the potential of BSPC-based PLBs to limit the interactions with specific inflammatory cells. This study is of paramount interest to the application of biosensors, which are largely silicon based. Such thin and stable BSPC-based PLBs can be applied as coatings on these sensors in an effort to overcome host response induced gradual loss of functionality.

METHODS AND MATERIALS

BSPC was prepared by modifying the procedure followed by Lamparski et al.¹⁴ 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.

Bilayer formation

Squares of silicon wafers (5 mm by 5 mm) were cleaned by sonication in acetone, ethanol, and ultrapure water three times for each. 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. BSPC was dried from the stock solution of chloroform with a stream of nitrogen. 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 ($T_m = 28.8^\circ\text{C}$).¹⁴ Aliquots of the vesicle solution were placed on the dried piranha treated wafers. Continuous bilayer coverage was attainable after 15 minutes. The substrates coated with lipids were carefully plunged into a shallow glass dish filled with ultrapure water for 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 a gentle stream of nitrogen. After polymerization the lipid coated wafers were sonicated with surfactant and then ultrapure water. The lipid-coated wafers were dried and stored under a nitrogen purged environment. 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° incident angle. Baseline readings were obtained from cleaned, bare silicon wafers. The refractive index of the lipid films was assumed to be 1.462 LGEMP software (Gaertner Scientific Corporation, Skokie, IL) was used to analyze and calculated thickness. 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.

Macrophage culture and analysis

The murine derived macrophage cell line, RAW 264.7, is a subclone of a line originally purchased from ATCC (Manassas, VA). 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°C , 5% CO_2 , and 95% humidity. To study macrophage behavior on the different surfaces, cells were seeded at a density of 2.5×10^4 cells/well onto the substrates placed in a 48-well plate. The cell number and phenotypic response was studied on PLB 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. Fluorescent images of the stained cell cultures were captured with a Nikon Eclipse model E-600 inverted microscope with Image Q software. 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 dendritic or spread cell and rounded cells was determined. A particle sorting and counting algorithm within the ImageJ program was used to distinguish between the two cases. This function allows the user to separate the different cells by their circularity and size. 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.

Statistical analysis

Statistical significance was determined by Student' *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.

RESULTS AND DISCUSSION

Ross et al. reported studies to optimize the polymerization conditions to generate highly uniform, stable bilayers of bis-SorbPC (structure shown in Fig. 1).²⁻³ In this study the optimized system was utilized to establish an *in vitro* system to address whether PLBs alter cellular attachment and morphology. Ellipsometric thickness and contact angle measurements for PLBs were used to confirm that proper deposition of the lipids on the wafers was established. In this analysis, each bilayer was sonicated in surfactant and then dried, which indicates the robustness of this system. The bilayer thickness is 4.3 ± 0.4 nm, and contact angle of the bilayer is $26^\circ \pm 3^\circ$ in contrast to the more hydrophobic bare silicon ($43^\circ \pm 2^\circ$). Our data is within the range of previously published data on redox PLBs of BSPC2 and the contact angle indicates that we have achieved a densely packed network of PC headgroups. The hydrophilic and zwitterionic nature of this system is crucial for the prevention of protein adsorption. Protein adsorption is a leading factor behind cellular attachment and behavior on any biomaterial surface.¹⁵ The protein resistant characteristics of lamellar lipid bilayers, phosphorylcholine modified polymers and PLBs of BSPC have been well studied previously.^{3,16-17} For example, Ross et al. showed that the surface coverage of nonspecifically adsorbed proteins on a BSPC PLB is about 1% of a monolayer.³ It was hypothesized that proteins are absorbed to defects in the PLB, not the PLB themselves.³ Knowing that protein absorption to PLBs is minimal, this study uses attachment and morphology of cells to test whether PLB coating can mediate the behavior of host response cells. The mechanism of cellular attachment to the PLB coated wafers seems to be twofold. First, proteins will adsorb to the limited number of defects present in the bilayer, and second the cells will attach to this small amount of adsorbed protein.

Macrophages play a crucial role in mediating the host response towards biosensors.¹⁵ When these sensors are implanted, the macrophage response initiates the early inflammatory response and later initiates a wound healing response. Activated macrophages begin to phenotypically differentiate early in the host response.¹⁸ They undergo a dendritic or spreading morphology to maximize the contact points with the surface. In biosensor applications, this can lead to failure or loss of functionality *in vivo*. It would be beneficial to have a biologically inert and stable coating that would limit cellular attachment. The goal of this project is to address whether PLBs could potentially function to mediate the host response.

RAW 264.7 macrophages are an excellent cell line that has many of the same phenotypic responses as primary macrophages.¹⁸ These cells were cultured onto PLB-coated or bare silicon wafers. As shown in Fig. 2A, the total number of cells on the PLBs at each time point was significantly below that of the bare silicon control. Representative images from the 24-hour culture (Fig. 2C) highlight the dramatic differences between the two conditions. Of the cells that were attached to the PLBs, very few displayed the dendritic morphology compared to the control samples. Similar behavior was also seen in a fibroblast culture completed

under the same conditions (see supporting information). The fibroblasts were analyzed due to their role in late stage of fibrous encapsulation of medical implants.

To quantify the morphological differences between PLB and silicon attached cells, the number of spread, or well attached cells, and rounded, or loosely attached cells, were enumerated using ImageJ analysis (Fig. 2B). The number of spread 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 PLB surface could be attaching to proteins adsorbed onto the defects in the PLB coating. We hypothesize that these defects are spaced far enough apart that the cells that do adhere cannot spread onto the surface and are in fact just clinging to a very small number of contact points. SEM analysis of cells on both PLBs and bare silicon revealed that the macrophages that attach to the bare silicon have a large number of filopodia extending in all directions from the cell (Fig. 2D), and at high magnification (Fig. 2E) each cell has a large number of contact points with the surface. As for the adherent cells on the PLBs (Fig. 2F-G) the number of contact points is much less than those on the control.

The limited number of attachment points that PLB-adherent cells correlates with our hypothesis that the cells are only able to attach to adsorbed protein in defects in the bilayer. Their rounded morphology suggests their inability to find sufficient attachment sites to support cell spreading. 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. The PLB coating prevents the initial cellular adhesion. This limited attachment could prevent the eventual encapsulation that is typical of the host response towards biosensors. We hypothesize that the coating will introduce minimal interference with the wound healing around the area that was insulted by the implantation of the biosensor. It would rather prevent the response directed at the surface of implanted biosensors.

Finally, a stability study was conducted on the PLB coated wafers by placing them in PBS under physiological conditions for up to 21 days. The bilayers had a slight decrease in thickness after 14 days, but maintained their hydrophilicity (Fig. 3A). The cause of the decrease in thickness could be due to the loss of small molecular weight segments of the bilayer. Despite the small changes in physical properties, when macrophages were cultured for 24 hours onto the PBS treated PLB coated wafers the cellular attachment and morphology was still comparable to PLB coated wafers not kept in PBS (Fig 3B-D).

CONCLUSIONS

The PLB coated wafers utilized in this study were found to be macroscopically uniform and within the ranges of other well-developed BSPC based PLBs. Our brief *in vitro* study showed that PLBs of BSPC coated on silicon substrates can limit attachment of specific cells involved in the host response to biosensors. The intrinsic non-adhesive property of this system that prevents cellular adhesion shows that PLBs could be an ideal platform for biosensor coatings where this behavior is beneficial. The PLBs analyzed in this work warrant further research into the mechanism of cellular attachment. Future *in vitro* studies will be conducted to examine the effectiveness of PLB coatings on inflammatory and wound healing responses. Specifically, intracellular cytokine staining will be employed to further identify the activation state of the attached cells. Also, AFM analysis will be conducted to better elucidate the true nature of the cellular attachment to the PLBs, and to help determine whether the attached cells are able to remodel the PLB coatings as a function of time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Chemical structure and theoretical polymerized structure of BSPC.

Figure 2.

(A) Total number of RAW 264.7 macrophage cells on PLBs (grey) and bare silicon (black) as a percentage of the control; (B) Percentage of cells that exhibit an spread morphology on PLBs (grey) and bare silicon (black). The PLB results exhibit statistical significance from each of the bare silicon data points ($p < 0.05$). (C) Fluorescent images of RAW 264.7 macrophages after 24 hours of culture on (left) PLBs and (right) bare silicon. Inset: * represents a cell that displays a rounded morphology, while # represents a cell that demonstrates a spread morphology. Scale bar = 100 μm . (D-G) SEM images of RAW 264.7 cells on bare silicon (D-E) and PLBs (F-G) after 24 hours of culture. (D, F: scale bar = 10 μm ; E, G: scale bar = 1 μm).

Figure 3.

(A) Ellipsometric thickness and water contact angle measurements of PLBs kept at physiological conditions for 0, 1, 4, 7, 14, and 21 days. (* designates statistical significance ($p < 0.05$) from the initial thickness). (B) Fluorescent images of a 24 hour culture of RAW 264.7 macrophages on bare silicon or PLB coated silicon samples after immersion for (left to right) 0, 1, and 21 day in PBS, and bare silicon (scale bar = 100 μm); (C) Total cell number as a percentage of control for PLB coated wafers immersed in PBS for 0, 1, and 21 day; (D) Percentage of spread cells for bare silicon, and PLB coated wafers immersed in PBS for 0, 1, and 21 day. Statistical significance is seen with all PLB data points in comparison with the bare silicon ($p < 0.05$).