Biostability and biocompatibility of a surface-grafted phospholipid monolayer on a solid substrate

Kwangmeyung Kim\textsuperscript{a}, Chulhee Kim\textsuperscript{b}, Youngro Byun\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a}Department of Materials Science and Engineering, Kwangju Institute of Science and Technology, 1 Oryong-dong, Puk-gu, Gwangju 500-712, South Korea

\textsuperscript{b}Department of Polymer Science and Engineering, Inha University, Inchon 402-751, South Korea

Received 17 February 2003; accepted 15 June 2003

Abstract

We have previously demonstrated phosphorylcholine monolayer chemically grafted onto a methacryloyl-terminated solid substrate by in situ polymerization. The in situ polymerization was carried out at the interface between a pre-assembled acrylated phospholipid monolayer produced by vesicle fusion and a methacryloyl-terminated substrate using a water-soluble initiator, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPD). Herein, we examined the biostability and biocompatibility of a surface-grafted phospholipid monolayer (poly-PC) on a methacryloyl-terminated substrate using a “wash off” test, in vitro protein adsorption and in vivo cage implantation for time intervals of 4, 7, 14 and 21 days, respectively. In order to compare the biostability and biocompatibility of phospholipid surfaces on solid substrates, we used two types of phospholipid surfaces: a physically adsorbed phospholipid monolayer (PC) and a poly-PC. Atomic force microscopy and water contact angle measurements indicated that the poly-PC surface was more stable in PBS, Triton X-100 and to EO gas sterilization than the PC surface. The adsorption of proteins such as albumin, fibrinogen, IgG and human plasma proteins on the poly-PC surfaces were significantly reduced, in vitro. Moreover, the poly-PC surface greatly reduced macrophage adhesion and the formation of foreign body giant cells, in vivo.

Keywords: Phospholipid monolayer; In situ polymerization; Biostability; Biocompatibility; Cytomimetic biomaterials

1. Introduction

Several kinds of artificial surfaces, which mimic the endothelial cell membrane, have been proposed [1,2]. Since outer cell membranes of endothelial cells are mainly composed of neutral phosphorylcholine-based lipids, the phosphorylcholine surface has been recognized as the best biologically inert surfaces. Because of its biocompatibility, several methods have been developed either to graft phosphorylcholine groups onto solid substrates or to synthesize various polymers containing phosphorylcholine groups [3–7]. Pidgeon et al. chemically grafted carboxylated phospholipids on an amine-terminated silica gel surface to form a stable phospholipid surface on solid substrates [3]. Ishihara et al. synthesized 2-methacryloyloxyethyl phosphorylcholine (MPC) copolymers with enhanced biocompatibility [4,5]. The biocompatibility of these phosphorylcholine modified surfaces has been improved by suppressing protein adsorption and platelet adhesion.

We have prepared a series of phospholipid monolayers using Langmuir–Blodgett (LB) or vesicle fusion methods [8,9]. These phospholipid monolayers self-assembled on solid surfaces in which the lipids can be controlled and easily assembled to a highly packed structure that could mimic the structure and biological function of cell membranes [10–13]. These phospholipid monolayers also showed improved biocompatibility, such as the prevention of protein adsorption and platelet adhesion. However, they showed limited stability and could be easily dissociated from the substrates due to physical adsorption of lipids on solid substrates.

In order to increase the stability of assembled phospholipid surfaces, many studies proposed polymerization of phospholipid assemblies containing reactive moieties, such as mono- or bis-acetylene, acryloyl, and sorbyl groups for increasing the stability of phospholipid assemblies [14–17]. The laterally polymerized
phospholipid surfaces exhibited acceptable stability under static conditions in water and air, as well as in the presence of high shear flow environment. However, the stability of polymerized phospholipid monolayers still needs to be increased in order to successfully apply these monolayers in clinical applications, because the polymerized phospholipids are associated with non-covalent interaction on solid substrates. Recently, we reported that an acrylated phospholipid monolayer was chemically grafted onto a methacryloyl-terminated substrate by in situ polymerization [18]. The polymerized phospholipid monolayer formed a surface-grafted lipid monolayer structure with high grafting efficiency, showing high stability to organic solvent.

In this study, we report on the biostability and biocompatibility of a phospholipid monolayer surface-grafted onto solid substrates. For this purpose, we prepared two types of phospholipid monolayer: a physically adsorbed phospholipid monolayer (PC) and a surface-grafted phospholipid monolayer (poly-PC). The biostability of PC and poly-PC surfaces were examined using a “wash off” test in PBS, Triton X-100 and EO gas sterilization. The biocompatibility of a poly-PC surface was evaluated by protein adsorption and cage implantation.

2. Materials and methods

2.1. Materials

1,12-dodecanediol, 2,6-di-tert-butyl-p-cresol, pyridine, 4-(N,N-dimethylamino)pyridine (DMAP), dicyclohexylcarbodiimide (DCC), and pyridinium dichromate (PDC) were obtained from Sigma Chemical Co. (St. Louis, MO). 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine was obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification. 3-(trimethoxysilyl) propyl methacrylate (TSM) was purchased from Gelest Inc. (Tullytown, PA). 2,2′-azobisis(2-methylpropionamidine) dihydrochloride (AAPD), chloroform and toluene were purchased from Aldrich Chemical Co. (Milwaukee, WI).

2.2. Preparation of an acrylated phospholipid and a methacryloyl-terminated substrate

An acrylated phospholipid, 1-stearoyl-2-[12-(acryloyloxy)-dodecanoyl]-sn-glycero-3-phosphocholine (acyrloyl-PC), and TSM-silanized silicon wafers and slide glasses were prepared as previously described [16,18]. Briefly, an acryloyl-PC was prepared by the esterification of 12-(acryloyloxy)-1-dodecanolic acid and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine. The product purity determined by 1H-NMR and TLC analysis was above 99%. The methacryloyl-terminated substrates were prepared by the silanization of TSM onto silicon wafers and slide glass surfaces. The cleaned silicon wafers and slide glasses (1 cm × 2 cm) were silanized in 2 wt% TSM/toluene solution at 80°C for 12 h. The TSM-silanized substrates were then washed with toluene, methanol and distilled water, respectively. They were dried under an argon atmosphere, followed by baking at 100°C for 12 h. Stability of the vinyl groups was confirmed by the peaks of FT-IR spectrum, C=O (1726.3 cm⁻¹) and C=C (1643.0 cm⁻¹) of the methacryloyl group on the TSM-silanized silica gels, reacted at 80°C for 12 h and followed by baking at 100°C for 12 h.

2.3. Preparation of a phospholipid monolayer on a methacryloyl-terminated substrate

A solution of acryloyl-PC in chloroform/methanol was placed in glass test tubes and the solvent was removed under a stream of argon. The produced acryloyl-PC film was rehydrated in distilled water (1 mg/ml) for 1 h and sonicated at 10 W for 10 min in an ice bath, thereby producing vesicles with the average diameter of 120 nm, as confirmed by dynamic light scattering (Malvern Instruments Ltd., Series 4700, Malvern, UK). An acryloyl-PC monolayer was then prepared by immersing a TSM-silanized substrate into the vesicle solution with gentle stirring for 3 h at room temperature. The supported phospholipid monolayer was washed with distilled water and dried under a stream of argon. This surface was used as a PC without in situ polymerization.

2.4. In situ polymerization of phospholipid monolayers on methacryloyl-terminated substrates

To make a poly-PC, a 1 wt% water-soluble initiator, AAPD, was added directly in glass test tubes containing lipid vesicles and methacrylated-terminated substrates after 3 h incubation. The glass test tubes were purged with argon before sealing with a rubber septum and then placed in an oil bath at 60°C for 15 min. After in situ polymerization, the substrates were washed with distilled water to remove AAPD. The polymerized acryloyl-PC monolayers were dried under an argon atmosphere. This polymerized acryloyl-PC monolayer was used as a poly-PC.

2.5. Biostability test of a poly-PC

In order to evaluate the biostability of PC and poly-PC surfaces, we performed a “wash off” test to Phosphate-buffered saline (PBS, pH = 7.4, ionic strength 0.15), Triton X-100 and EO gas sterilization. The PC and poly-PC surfaces were incubated in PBS and 1 wt% Triton-X100 solution in PBS for 2 h at 37°C, respectively, and they were also sterilized with EO gas at 55°C.
for 6 h. After incubation in PBS or Triton X-100 and EO gas sterilization, the samples were washed with distilled water to remove buffer salts, Triton X-100 and EO gas. They were then dried under an argon atmosphere. The biostability of PC and poly-PC surfaces to each “wash off” test was examined using water contact angle measurements and atomic force microscopy (AFM).

2.6. Characterization

AFM images were obtained in air at room temperature using an Autoprobe CP system (Park Scientific, Inc., Sunnyvale, CA). Contact mode images were made using a contact silicon ultralevers with a spring constant of 0.06 N/m. AFM images were analyzed by using PSI Proscan Software (Park Scientific, Inc.). Images presented were obtained after several repetitions of the samples, which revealed similar features with regard to the depth of holes and the root-mean-square (rms) roughness. The surface coverage of acryloyl-PC molecules on PC and poly-PC surfaces was determined by performing the bearing analysis (PSI Proscan, Park Scientific, Inc.). The static water contact angle of samples was measured by a contact angle goniometer (Rame-Hart, Inc.). All measurements were carried out at room temperature and 40% humidity.

2.7. Protein adsorptions, in vitro

The amount of proteins adsorbed on the samples was quantified using micro-bicinchoninic acid (m-BCA) protein assay (Product No. 23235, Pierce Chemical Co., Rockford, IL) [19]. Protein adsorption was performed in a closed adsorption cell (Falcon tube, 15 ml). PBS was used to prepare solutions of each individual protein (100 µg/ml), such as human serum albumin (HSA), human plasma fibrinogen (Fg) and human serum IgG, respectively. The human plasma was obtained from unmediated, adult volunteers as previously described and diluted to 10 v/v% in PBS [8]. Each sample was placed vertically in the adsorption cell. Then, the adsorption cell was filled with PBS and left for 1 h to hydrate, after which the cell was quickly refilled with 3 ml of each protein and human plasma solution. Protein adsorption was then allowed to take place for 2 h at 37°C. After protein adsorption, samples were flushed with PBS and then slightly rinsed with PBS to remove the weakly adsorbed proteins. The adsorbed proteins were then eluted by sonication (with the total surface area of 12 cm²) in 1 ml of 1 wt% sodium dodecyl sulfate (SDS) solution for 1 h. The eluted protein solution was quantified with a Microplate Reader (VERSAnax, Molecular devices) at 562 nm, a value based on a calibration curve obtained with bovine serum albumin standard solution (Sigma).

2.8. Cage implantation, in vivo

Cages were constructed and implanted as previously described [20]. Stainless-steel mesh was formed into a 12 mm-diameter and 25 mm-length tube. The PC and poly-PC samples on slide glasses were EO gas sterilized and placed inside the cages at one sample per cage. Two cages for each of samples were implanted subcutaneously on the back of the Sprague–Dawley rat (250–300 g). After 4, 7, 14 and 21 days of implantation, the rats were sacrificed and the cages were removed. The samples were then removed from the cages and immediately rinsed in distilled PBS and stained with modified Wright’s stain to evaluate macrophage density and foreign body giant cell (FBGC) formation with an optical microscope (Nikon diaphot 300, Japan). Quantitative measurements were carried out from 10 representative areas per specimen.

2.9. Statistics

All data were statistically analyzed by a paired t test at 95% confidence level (p < 0.05) and were reported as Mean ± SD.

3. Results

3.1. AFM images of PC and poly-PC surfaces

Because the TSM-silanized surfaces had a very flat surface with the rms roughness of 1.6 Å, which was very similar to that of bare silicon oxide surface, the thickness and surface density of PC and poly-PC could be measured by using line profile analysis and the bearing ratio of each AFM image [18]. Fig. 1 shows the AFM images of PC and poly-PC surfaces on solid substrates. The PC surface showed a smooth surface with the rms roughness of 2.1 Å and its average thickness from the monolayer surface to the hole bottom was 2.9 nm. The surface coverage of acryloyl-PC molecules on the PC surface was more than 93%, indicating that the hole defects with the depth of 2.9 nm was less than 7% in the monolayer. The poly-PC surface showed a very flat surface with the rms roughness of 1.8 Å and the average thickness of 3.0 nm. The surface coverage of acryloyl-PC molecules on the poly-PC surface was above 95%. Schematic representations of PC and poly-PC surfaces were drawn from the AFM measurements.

3.2. Biostability of PC and poly-PC surfaces

The biostability of PC and poly-PC surfaces were examined using water contact angles (Fig. 2) and AFM measurements (Fig. 3). The TSM-silanized silicon surfaces had a hydrophobic surface of methacryloyl
groups on the substrate as control. In the case of PC and poly-PC surfaces, the water contact angles decreased to about 54° due to the hydrophilic phosphorylcholine surfaces. After a “wash off” test in PBS, Triton X-100 and EO gas sterilization, the surface coverage of phospholipid molecules was quantified using bearing ratio of each AFM image. The PC surfaces had different surface coverage of 71%, 5% and 32% with respect to PBS, Triton X-100 and EO gas sterilization, respectively. But the poly-PC surfaces showed a high stability in PBS, Triton X-100 and EO gas sterilization. The surface coverage of poly-PC surfaces was 95%, 90% and 92% in PBS, Triton X-100 and after EO gas sterilization, respectively. The poly-PC surfaces still maintained the monolayer structure with some hole defects after the “wash off” test.

3.3. Protein adsorptions on PC and poly-PC surfaces

Using a m-BCA protein assay kit, protein adsorption on the PC and poly-PC surfaces was evaluated after they had come in contact with human proteins such albumin, fibrinogen, IgG and human plasma proteins. The amounts of adsorbed proteins on PC and poly-PC surfaces are shown in Fig. 4. Protein adsorptions to TSM-silanized substrates were higher (p<0.05) than that of both PC and poly-PC surfaces. In particular, the poly-PC surfaces showed a very low protein
adsorption, compared to TSM-silanized substrates and PC surfaces.

3.4. Cage implantation for PC and poly-PC surfaces

Fig. 5 shows the morphology of macrophages and FBGCs on PC and poly-PC surfaces at 4, 7, 14 and 21 days of implantation, respectively. We also included optical micrographs of the TSM-silanized glass substrates as control. At 4 days, monocyte-derived macrophages were the predominant cell type in the inflammatory response in all samples. TSM-silanized glass substrates and PC surfaces produced high density of macrophages, but poly-PC surfaces showed a low surface density of adhered macrophages. By day 7, the cells were less dense, and smaller FBGCs, generally containing 10–20 nuclei, were occasionally observed on TSM-silanized substrates and PC surfaces. The poly-PC surfaces also showed a few FBGCs and the loss of macrophages from the surface. By 14 days, the TSM-silanized substrates and PC surfaces showed high surface density of large FBGCs,
whereas the poly-PC surface exhibited low-surface density of FBGCs. By 21 days, the TSM-silanized substrates and PC surfaces exhibited a highly dense coverage of an FBGC monolayer, but the poly-PC surfaces greatly suppressed the FBGCs formation on the surface.

The number of macrophages adhering to TSM-silanized substrates, PC and poly-PC surfaces as a function of implantation periods is shown in Fig. 6. TSM-silanized substrates and PC surfaces attracted a similar quantity of macrophage regardless of implantation period, even though PC surfaces were modified with
acryloyl-PC. The poly-PC surfaces resisted the adherence of macrophages during the first 4 days, compared to both TSM-silanized substrates and PC surfaces. Otherwise, all samples showed similar macrophage density at 7, 14 and 21 days.

In contrast to the macrophage density, the FBGC density on samples was minimum at 7 days, when it rapidly increased up to 21 days (Fig. 7). It is worth noting that the FBGC density was significantly lower \( p < 0.05 \) on poly-PC surfaces up to 21 days than both TSM-silanized substrates and PC surfaces. The FBGC density on both TSM-silanized substrates and PC surfaces was three times higher than that on poly-PC surfaces (e.g. 600 ± 780 FBGCs/cm² on TSM-silanized substrates, 650 ± 782 FBGCs/cm² on PC surfaces, and 220 ± 200 FBGCs/cm² on poly-PC surfaces).

The average FBGC size indicated that TSM-silanized substrates and PC surfaces had an attractive surface for FBGC formation, on which the FBGC size rapidly increased from 0.002 to 0.006 mm² at 7 days to 0.04–0.042 mm² at 21 days (Fig. 8). In comparison, a significantly lower FBGC size on poly-PC surfaces was observed for the whole implantation period. The maximum size of FBGC on poly-PC surfaces was about 0.01 mm², 24–25% of the average FBGC size on TSM-silanized substrates and PC surfaces at 21 days.

4. Discussion

We have previously reported that a surface-grafted phospholipid surface was successfully prepared using in situ polymerization [18]. Under the optimized polymerization conditions with a 1 wt% AAPD initiator, the polymerized phospholipid surface showed a flat and smooth monolayer structure of phosphocholine lipids. The XPS study further confirmed that the polymerized phospholipid had a high grating efficiency of 94.5% and that it formed a stable phospholipid monolayer chemically grafted to a solid substrate.

It has been well known that self-assembled phospholipid surfaces offer a high level of biocompatibility [9,21]. However, the main shortcoming with the strategy in mimicking cell membranes is the lack of stability
stemming from physical coating. This is the reason why we introduced the in situ polymerization of a pre-assembled phospholipid monolayer to solid surfaces. In this study, AFM images showed that the PC and poly-PC surfaces had a very flat surfaces with the rms roughness of less than 2 Å, whereas the rms roughness of a cleaned silicon wafer and a TSM-silanized silicon substrate was about 1.5 Å. The average thickness of PC and poly-PC surfaces was about 3 nm, which was very similar to the length of one molecule of acryloyl-PC (3.6 nm).

In the “wash off” test, we confirmed the biostability of PC and poly-PC surfaces to PBS, Triton X-100 and sterilization using water contact angle and AFM measurements. Phospholipid monolayers have been prepared on hydrophobic surfaces through formation of monolayer of alkyl chains and the phospholipid molecules are held onto the surface solely through weak hydrophobic interactions [16–18]. In physiological conditions, the lipid monolayer was easily detached and it could not offer enough stability for medical applications. However, the poly-PC surface showed high stability in PBS, surfactant and sterilization when compared with PC surfaces. The poly-PC surface still formed a phospholipid monolayer structure with a high coverage above 90% after exposure to PBS, surfactant and sterilization. As expected, physically adsorbed phospholipid monolayers showed low stability in surfactant and sterilization. The physically adsorbed phospholipid molecules were easily removed by exposing them to PBS, surfactant and sterilization.

The biocompatibility of phospholipid surfaces was closely related to biostability of phospholipid surfaces. PC surfaces decreased the in vitro protein adsorption more than the hydrophobic control surface, but the degree of prevention was less than that of poly-PC surfaces. These results indicated that the biocompatible phospholipid molecules are removed from the substrate during protein adsorption in PBS for 2 h at 37°C and the exposed defects activate the protein adsorption, as predicted from AFM measurements (Fig. 3a). Chemically grafted phospholipid polymer surfaces reduced the protein adsorption to the detection limit level of 0.5 μg/ml which was a low protein adsorption as reported for highly packed phospholipid surfaces [22].

In vivo cellular responses to PC and poly-PC surfaces give rise to an apparent relationship between biostability and biocompatibility of phospholipid surfaces. Although PC surfaces were modified with biocompatible phospholipid monolayers, they did not present biocompatibility in vivo cage implantation. Because, by repeating washing and sterilization, the physically adsorbed phospholipid molecules should be removed from the solid surface, in vivo. Therefore, we did not evaluate the biocompatibility of assembled phosphocholine groups’ surfaces, in vivo. On the other hand, the poly-PC effectively suppressed macrophages adhesion within 4 days and FBGCs formation on solid surface up to 21 days. This is because the stabilized phosphocholine groups on poly-PC surfaces could resist the inflammation reaction of macrophages and FBGCs [23,24].

We conclude that the surface-grafted phospholipid monolayer, which mimics the structure and biological property of cell membranes, was successfully prepared using in situ polymerization, and this surface was stable, and suppressed the protein adsorption and foreign body reaction.

Acknowledgements

This work was supported by Korea Research Foundation, and partially supported by BK21 program in Korea.

References


