Combined study of X-ray reflectivity and atomic force microscopy on a surface-grafted phospholipid monolayer on a solid

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Abstract

We investigated the detailed structure of a surface-grafted phospholipid monolayer, which was polymerized in situ onto a methacryloyl-silanized solid surface. By the combined study of X-ray reflectivity and atomic force microscopy, the in situ polymerization step of the lipid molecules are sufficiently detailed to reveal the molecular structure of lipid molecules before and after in situ polymerization. From the data of the X-ray reflectivity, we confirmed that the in situ polymerization process produces a flat lipid monolayer structure and that the lipid monolayer is substantially grafted on a silanized surface by chemical bonding. After the polymerization and washing processes, the thickness of the head group was 9 Å and the thickness of the tail group was 21 Å. The surface morphology of the polymerized phospholipid monolayer obtained by the measurements of atomic force microscopy was consistent with the results of the X-ray reflectivity. The cross-sectional analysis shows that the surface coverage of lipid molecules, which are chemically grafted onto a silanized surface, is approximately 89%.

Keywords: Phospholipid monolayer; In situ polymerization; X-ray reflectivity; Atomic force microscopy

1. Introduction

Supported phospholipid membranes on solids, particularly lipid bilayers and monolayers, are useful model surfaces for the characterization of many important biological processes. Moreover, interest is growing in the potential application of supported membranes as biosensors, pharmaceutical screening, some kinds of signal devices, and medical implant applications [1–4]. Phospholipid monolayers and bilayers on solids are commonly prepared using the Langmuir–Blodgett (LB) technique or the vesicle fusion technique [5–9]. These lipid membranes have the advantage of being easy to use and they can accurately mimic the lipid head group surfaces of cell membranes. However, the limited stability of phospholipid membranes on solids, which is due to physically adsorbed phospholipid molecules, is an obvious drawback. This limited stability gives rise to the following major disadvantages:

• reorganization of lipid head group structures on solids is expected in air (hydrocarbon prefers the side that contains air) [5];
• an inhomogeneous surface can be formed due to multilayered lipid structures or large vesicle adsorption during the LB deposition or vesicle fusion process [8];
• phospholipid assembles cannot offer enough stability to surfactant and organic solvents [9].

To increase the stability of phospholipid monolayers on solids, Chaikof and co-workers introduced in situ polymer-
ization of phospholipid monolayers that contain monoacryloyl groups on solids [10,11]. Laterally polymerized phospholipid monolayers are produced in acceptable stability under static conditions in water and air and in the presence of a high shear flow environment; however, a significant amount of lipids was desorbed by being exposed to surfactant and organic solvents. Recently, we reported a method of preparing a surface-grafted phospholipid monolayer using in situ polymerization at the interface between a monoaoylated lipid monolayer and a methacryloyl-terminated surface [12]. As confirmed by studies on water contact angles, atomic force microscopy (AFM), and X-ray photoelectron spectroscopy (XPS), a polymerized phospholipid monolayer (poly-PC) can be chemically bonded to a solid in a substantial manner and remain stable to organic solvents. The reduced adsorption of proteins and macrophages on the poly-PC can be clearly observed by in vivo experiments, as well as by in vitro experiments [13].

Previous studies on vesicle fusion and the in situ polymerization step of lipid molecules lack sufficient detail to reveal the molecular structure of the lipid molecules; for instance, whether the polymerization produces the monolayer structure of the lipid molecules and whether there is any influence on the structure of the adsorbed lipid molecules due to the polymerization. For the potential application of a surface-grafted phospholipid monolayer, it is crucial to determine the detailed physical structure of the lipid monolayer grafted on solids. The studies described here are therefore concerned with a depth profiling measurement that characterizes the surface properties of phospholipid surfaces before and after in situ polymerization. By modeling and refining the density profiles, we used X-ray reflectivity (XR) in particular as a powerful method of quantitatively evaluating various structural analyses such as thickness, density, and roughness [14–16]. We then took AFM measurements after each vesicle fusion and in situ polymerization process.

2. Materials and methods

2.1. Materials

We purchased TSM (3-(trimethoxysilyl)propyl methacrylate) from Gelest Inc. (Tyllytown, PA, US), and we purchased AAAPD (2,2’-azobis-(2-methylpropionamidine) dihydrochloride), methanol, and toluene from the Aldrich Chemical Co. All solvents were distilled and stored over 3-Å molecular sieves before use. We then prepared an acryloyl–PC monolayer by immersing a TSM-silanized substrate in the vesicle solution with gentle stirring for 3 h at room temperature. After washing the supported phospholipid monolayer with distilled water and drying it under a stream of argon, we used this surface as a PC without in situ polymerization. To make a poly-PC, we added a 1 wt% water-soluble initiator, AAPPD, directly into glass test tubes containing lipid vesicles and methacryloyl-terminated substrates that had been incubated for 3 h. We purged the glass test tubes with argon before sealing them with a rubber septum and then placed them in an oil bath at 60°C for 15 min. After in situ polymerization, the substrates were washed with distilled water to remove the AAPPD. We dried the polymerized acryloyl–PC monolayers in an argon atmosphere and used the polymerized acryloyl–PC monolayer as a poly-PC (Fig. 1c). For our XR and AFM studies, we used these different surfaces of the TSM-silanized substrate, PC and poly-PC.

2.2. Preparation of a surface-grafted phospholipid monolayer

A surface-grafted phospholipid monolayer on a methacryloyl-terminated substrate was prepared as described elsewhere [12,13]. Briefly, we prepared the methacryloyl-terminated substrates by silanizing TSM onto silicon wafers. We then silanized the cleaned silicon wafers (1 x 2 cm) 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 (Fig. 1a). We placed a solution of acryloyl–PC in chloroform/methanol in glass test tubes and removed the solvent under a stream of argon. We then rehydrated the produced acryloyl–PC film in distilled water (1 mg/ml) for 1 h and sonicated the film at 10 W for 10 min in an ice bath, thereby producing vesicles with an average diameter of 120 nm. As shown in Fig. 2b, these results were confirmed with the aid of dynamic light scattering (Malvern Instruments Ltd., Series 4700, Malvern, UK).

We then prepared an acryloyl–PC monolayer by immersing a TSM-silanized substrate in the vesicle solution with gentle stirring for 3 h at room temperature. After washing the supported phospholipid monolayer with distilled water and drying it under a stream of argon, we used this surface as a PC without in situ polymerization. To make a poly-PC, we added a 1 wt% water-soluble initiator, AAPPD, directly into glass test tubes containing lipid vesicles and methacryloyl-terminated substrates that had been incubated for 3 h. We purged the glass test tubes with argon before sealing them with a rubber septum and then placed them in an oil bath at 60°C for 15 min. After in situ polymerization, the substrates were washed with distilled water to remove the AAPPD. We dried the polymerized acryloyl–PC monolayers in an argon atmosphere and used the polymerized acryloyl–PC monolayer as a poly-PC (Fig. 1c). For our XR and AFM studies, we used these different surfaces of the TSM-silanized substrate, PC and poly-PC.

2.3. X-ray reflectivity

We used XR to monitor the structural changes of lipid layers during the vesicle fusion and in situ polymerization process. The measurements were taken at the 5C2 K-JIST beamline of the Pohang Accelerator Laboratory when the energy was 10 keV, which corresponds to a wavelength of λ = 1.24 Å. We defined the specular reflectivity as the intensity taken as a function of the wavevector transfer, qz, by varying the incident (αi) and exit (αf) while maintaining αi = αf. Thus, qz is given by qz = (4π/λ)sinαi. Because the specular reflectivity detects the variation of the electron density ρ(z) in the direction of a normal surface averaged in a parallel plane to the sample surface, the specular reflectivity is sensitive to the layer thicknesses (d), the density contrasts and the interfacial roughness (σ) defined by the probability density. To evaluate the goodness of fit, we used
a least square algorithm. During the fitting process, we systematically varied and then optimized the fitting parameters \( \rho, d, \) and \( \sigma \) until we minimized the goodness of fit, \( \chi^2 \). In this study, the XR shows enough resolution to determine the polymerized acryloyl–PC layer within a few angstroms. The typical thickness error is 2 to 3 Å and the typical roughness error is 10 to 15% of the value.

2.4. AFM measurements

Using an Autoprobe CP system (Park Scientific, Inc., Sunnyvale, CA), we obtained AFM images in air at room temperature, and we used contact silicon ultralevers to make contact mode images. We determined the surface coverage of surface-grafted lipid molecules with the aid of cross-sectional analysis, in which we measured the ratio of the material filled length to the evaluation length at an intersection line (PSI Proscan) [18].

3. Results and discussion

Fig. 2a shows the XR results from the spatial evolution of the TSM-silanized substrate (open circles), PC (closed circles), and poly-PC (open triangles). Note that large footprint of the incoming X-ray beam at very small angles reduces the total reflection amplitude to less than 1. First, the XR curve of a TSM-silanized substrate exhibits only a large modulation (the Kiessig fringe), indicating that on oxide surfaces with a thickness of 11 Å the TSM-silanized surface has a uniform thickness of \( d = 10 \) Å. The persistence of the Kiessig fringes to large \( q_z \) values is consistent with the small surface roughness between the TSM-silanized substrate and
the air obtained from the fits, $\sigma = 3 \, \text{Å}$. The smooth interface indicates that a highly packed TSM monolayer has been produced.

Next, we evaluated the PC surface on the TSM-silanized substrate. At a glance, the XR curve had irregularly spaced multiple dips, implying that in this step the preassembled acryloyl–PC film had formed a multilayer structure. To describe the detailed structures, a feasible electron density profile perpendicular to the film layer was initially modeled and refined repeatedly until it matched the experimental reflectivity curve [19,20]. Note that a number of solutions can be found due to the phase problem. Of these, we identified the most likely solution when we obtained a physically reasonable model which fitted the experimental data and which supported other data such as the AFM and XPS measurements [12,23].

We first calculated the ideal density profile from the published density values as an ideal layer structure. As previously established by numerous groups [15,16], our phospholipid layer model consisted of two constant density parts: one corresponding to a polar head region ($\rho_{\text{head}} = 0.476 \, \text{e}^- / \text{Å}^3$) and the other corresponding to a hydrophobic hydrocarbon tail region ($\rho_{\text{tail}} = 0.324 \, \text{e}^- / \text{Å}^3$). Depending on their electron densities, the indices of refraction of the tail and head group layers differ sufficiently. For example, if we neglect absorption, the calculated indices of refraction $n = 1 - \delta$ at an incident energy of 10 keV with ideal dispersion $\delta_0$ of the tail and head regions are given by $n_{\text{tail}} = 1 - 2.254 \times 10^{-6}$ and $n_{\text{head}} = 1 - 3.310 \times 10^{-6}$, respectively.

To obtain a suitable fitting, we therefore used three adjustable parameters for each repeating bilayer structure: the thickness of the head groups and aliphatic tails, their respective dispersion $\delta$, and the roughness at the boundaries. Using recursive Parratt formalism, we then varied these values systematically to get the best fitting with reasonable variation of the density, roughness, and thickness of each layer [21]. Quite often, for systems with very thin layers (such as the head regions), the surface roughness is bigger than the thickness of its underlying layer. In this case, the total density profile produces a discontinuous model profile that is unrealistic in nature [22]. To generate continuous model profiles, we used the effective-density model [22]. In contrast to the classical box model, each layer profile of the effective-density method is sliced into very thin layers (up to 1 Å) with uniform dispersions and sharp interfaces. Fig. 2b shows that a reasonable fit occurs over the entire $q$-range, which is shown as a solid line; it also shows the refined model of the acryloyl–PC multilayer (closed circles).

As shown in the figure, the density profile reveals that the system has at least three high-density regions, which are considered the head-to-head layers ($h_1$, $h_2$, and $h_3$ in Figs. 2b and 2c). The layers consist of two head groups from the upper and bottom PC layers of the model in Fig. 2c. The profile also reveals the formation of two low-density hydrocarbon regions between the higher density regions. Note that the $y$-axis in the model profiles (Fig. 2b) represents $\delta$, and the parameter of $\delta$ is given linearly in terms of the electron density of $\rho$ by $\delta = \lambda^2 \rho_0 / 2\pi$, where $\rho_0$ is the classical electron radius. The first high-density region ($h_1$) was modeled with approximately 84% of the fully packed polar part and its thickness, 25 Å, was comparable to the head-to-head region of two phospholipid layers. The packing density decreased for the second high-density region ($h_2$) by 75%, which is indicative of an incomplete ordering. Fig. 2c depicts schematically represents the PC multilayer model, which is based on the density profile. After the acryloyl–PC multilayer was polymerized by adding the initiator (AAPD) into the system, the change in the overall reflectivity curve was minor. Hence, only negligible structural changes resulted from the in situ polymerization process (from the steps of Fig. 1b to Fig. 1c).

After washing the PC and poly-PC surfaces with methanol, we carefully compared them to determine the polymerized effects on the first lipid layer that contacts the TSM molecules. First, the methanol-washed, nonpolymerized PC surface (data is not shown) showed nearly the same XR profile as the TSM-silanized substrate. The similar profiles indicate that the physically stacked multilayers on the TSM surface, including the first nongrafted lipid layer, are completely detached during the washing process.

Fig. 2. (a) X-ray reflectivity for a TSM-silanized substrate ( ), PC ( ), poly-PC ( ), and methanol-washed poly-PC ( ) and their fits (lines). The curves are vertically shifted on the reflectivity scale with respect to each other for clarity. (b) Corresponding density profiles of the PC ( ) and methanol-washed poly-PC ( ) are displayed. (c) Schematic diagram representing the PC multilayer model ( ) in (b).
After each preparation step, we thoroughly rinsed the substrates with distilled water to remove any remaining solvent or organic initiator. Nonetheless, because we wondered whether any solvents were retained in the layers after the washing process, we dried the substrates further in an argon atmosphere. However, we could not completely rule out any insignificant amount of adsorption from atmospheric moisture during the measurement process because we had taken all the measurements under normal ambient conditions.

The methanol-washed poly-PC film showed a unique reflectivity curve (the closed triangles shown in Fig. 2a). The curve differs significantly from that of the TSM-only surface and the PC multilayer insofar as it still shows oscillations from the monolayers of the poly-PC and TSM molecules. The multiple Bragg peaks observed previously at the poly-PC surface have disappeared, indicating that the multilayered phospholipid molecules were successfully washed off.

The “packing” structure of the surface-grafted lipid layer can be determined by the thickness and segment volume fraction. We first deduced the tilt angle of the grafted phospholipid to \( \sim 34^\circ \) given by \( \cos \theta = l_{\text{tail}}/l_{\text{max}} \), where the \( l_{\text{max}} \) is the fully extended molecular length of an aliphatic tail, which is given by \((1.5 + n \times 1.265) \) Å. Importantly, the acryloyl–PC molecule consists of two asymmetric tails, a fully saturated \( \text{C}_{16} \) and an acryloyl-functionalized \( \text{C}_{12} \). The asymmetric tails can influence the structure of the polymerized PC layer. Various references to Langmuir (or LB) monolayers suggest a tilt angle of \( 30^\circ \) for most phospholipid membranes [16]. By assuming the close packing of the electron density of \( \text{C}_2 \) groups in the tails of 0.324 \( \text{e}^-/\text{Å}^3 \), we estimated that the packing density of the grafted acryloyl–PC layer was 87\%. In detail, the volume fraction of the tail region of the grafted acryloyl–PC layer (\( \rho_{\text{tail}} \) in Fig. 2b) was approximated by \( \rho_{\text{tail}} = \int_0^{l_{\text{max}}} \rho_{\text{tail}}(z) \, dz / \int_0^{l_{\text{max}}} \rho_{\text{max}}(z) \, dz \), where \( \rho_{\text{tail}}(z) \) is the experimental electron density profile and \( \rho_{\text{max}} \) is the ideal electron density of a fully saturated tail part, which is given by a step function [16,24]. \( z \) is the size of the system in the direction perpendicular to the substrate. For a fully saturated \( \text{C}_{16} \), \( l_{\text{max}} \) was 21.7 Å thick. This estimation indicates successful polymerization between the acryloyl–PC layer and the TSM molecules. Hence, the washing process failed to desorb any significant amount of the grafted acryloyl–PC molecules.

The thickness of the polar phospholipid group was 9 Å, which is about half the thickness of the head-to-head polar layer (\( h_{1} \)). The head group surface, however, was not completely smooth after the washing process. This lack of smoothness reduced the \( \delta_{\text{head}} \) at the mid plane of the head group region to \( \delta_{\text{head}1} \sim 2.05 \times 10^{-6} \) with a relatively large level of roughness, \( \sigma = 9 \) Å. Moreover, reasonable fits over the entire \( q \)-range required the inclusion of a 20-Å-thick low-density layer (\( \delta_{\text{head}2} = 0.476 \times 10^{-6} \)) on the head group surface. Thus, the model in Fig. 2b consists of two headgroup layers: a high-electron-density layer (head 1) adjacent to the tail region and a second layer of lower electron density at the free surface (head 2).

We can infer from the difference between the packing densities of the tail region and the head region with a diffuse interfacial structure that the poly-PC molecules do not homogeneously rise straight up from the TSM surface. A fraction of the polymerized phospholipid molecules might lie horizontally on the nonoccupied TSM surface. As a result, the average density of the tail region remains high due to the presence of head groups, whereas the average density of the head region decreases significantly. The layer head 2 has a much lower density, comparable to the density of 10 to 20\% of the nonpolymerized residue molecules. Some of the acryloyl–PC molecules might not be completely fixed on the surface (as in the case of incomplete polymerization), and they might dangle from the surface with more local freedom. In that case, the head groups of the incompletely polymerized PC molecules can protrude from the level of the grafted PC molecule surface. Table 1 summarizes the detailed dimensions of the phospholipid layers from the X-ray analysis.

We also used AFM measurements to study the lateral structure of PC and poly-PC surfaces. The TSM-silanized surfaces had a very flat surface with an rms roughness of 2 Å, which agrees well with the XR results [12]. We measured the thickness and surface density of the PC and poly-PC by using line profile analysis and the bearing ratio of each AFM image. After the vesicle fusion, the PC surface shows uneven morphologies composed of a phospholipid monolayer

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<th>Samples</th>
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<td>Poly-PC</td>
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<td>Methanol-washed poly-PC</td>
<td>21</td>
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<sup>a</sup> The thicknesses of the tail and head regions were obtained from the first tail layer (\( \delta_{\text{tail}} \) in Fig. 2b) that was in contact with a TSM substrate, and its head group layer.

<sup>b</sup> All tilt angles were calculated from the ratio of the first tail length to the fully extended tail length.

<sup>c</sup> All packing densities were calculated from the electron densities of the first tails to the close packed electron density of the \( \text{CH}_2 \) group.

<sup>d</sup> These values include both contacting head groups, one from the first PC and the other from the second PC layer.

Note: The typical thickness error is between 2 and 3 Å.
and multilayer structures (Fig. 3a). There were also many vesicles with a diameter of about 100 nm. In addition, the maximum thickness of 115 Å measured by a line profile inset image indicates the formation of a lipid multilayer; we also found monolayer steps with a thickness of 30 Å. This result substantiates the notion that normal techniques of vesicle fusion yield a very rough lipid surface—a consideration that could significantly affect any membrane-related studies. Again, this structure is consistent with the XR result described above. The poly-PC surface, which shows uneven morphologies similar to those on the PC surface, contains lipid multilayers and fused vesicles (not shown here).

After washing the poly-PC surface with methanol, we found a fairly homogeneous PC monolayer on a TSM-silanized substrate (Fig. 3b). We also observed a number of holes and partially roughened areas in the upper area of the figure. A comparison of these data with the XR data shows that the physically adsorbed lipid molecules and vesicles were successfully removed. The average thickness of the grafted monolayer was 30 Å, which is consistent with the XR results. The dimensions of the surface roughness and the diffused structure were also comparable to the XR results. The cross-sectional analysis shows that the surface coverage of lipid molecules was approximately 89%. Again, this result agrees well with the XR study for the quantitative analysis of the packing density of the tail layer. Previously we showed that the grafting efficiency of an acryloyl–PC monolayer on the TSM substrate was 94.5%—for this calculation, which is based on the relative carbon ratio of the poly-PC layer, we used an XPS technique before and after the washing process [12]. Overall, we estimated the production of about 6% of defects during the vesicle fusion and another 6% of defects during the polymerization and washing process. By combining XR and AFM results, we made a schematic model for PC and methanol-washed poly-PC surfaces, as shown in each AFM image.

4. Summary

Our combined study of XR and AFM reveals the detailed structures of a phospholipid surface before and after in situ polymerization. We successfully grafted a polymerized phospholipid to a methacryloyl-terminated surface and we removed the uneven lipid multilayers and vesicles by methanol washing. From the XR data, we show that the polymerization process produces a very flat lipid monolayer structure and that the lipid monolayer is stabilized on a silanized surface by chemical bonding. After the polymerization and washing processes, the thickness of the head group was 9 Å and the thickness of the tail group was 21 Å. This work clearly demonstrates that in situ polymerization techniques can produce a stabilized phospholipid surface. These techniques therefore offer a great opportunity for creating an ideal and versatile membrane surface that can be used at the air–solid interface and the liquid–solid interface at any given shear condition.

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