Fabrication of Supported Lipid Bilayer on Graphene Oxide

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Abstract. Planar lipid bilayers of dioleoylphosphatidylcholine was prepared on graphene oxide (GO), as a fundamental platform for biosensing in plasma membrane model using graphene. The GO flakes were prepared according to modified Hummer’s method, and deposited on thermally oxidized SiO₂/Si surfaces. We found that planar lipid bilayers were reproducibly formed on the GO/SiO₂/Si surface in the presence of Ca²⁺ ions, while unruptured vesicles remained on the GO surface without Ca²⁺ ion. The results of atomic force microscope observation and fluorescence recovery after photobleaching experiment revealed that double lipid bilayers were spontaneously formed on the GO surfaces.

1. Introduction
A lipid bilayer is the fundamental structure of plasma membranes. The lipid bilayer is not only a static wall between the inside and outside of cell, but also has important functions, such as the transportation of small molecules, ions, and signals into and out of cells through specific channels and endo- and exocytosis, through the two-dimensional organization and molecular diffusion [1,2]. The behavior of lipids and proteins in plasma membranes and their relation with the functions during reactions have been studied extensively [2-4]. Supported lipid bilayers (SLBs), which are artificial bilayer membrane at solid-liquid interfaces, have been investigated as cell membrane models to study the physicochemical properties of lipid bilayers, and as the platform for membrane proteins [5,6]. Recently, some new methods were reported to obtain detailed information of biochemical molecules using graphene and graphene oxide (GO) [7-11]. Our purpose is the development of a new method to obtain detailed information of biomolecules on and in plasma membranes, using graphene-supported lipid bilayers (G-SLBs). We prepared the SLB of phosphatidylcholine on GO by the vesicle fusion method [12-16].

At the formation of SLB by the vesicle fusion method, the efficiency of the formation of planar membranes depends on the material, shape and chemical states of substrate surfaces [15-17]. It is necessary to establish a reproducible condition for the formation of SLB on GO. GO has amphiphilicity, because GO consists of the hydrophilic sp³-carbon regions including –OH, –O–, and –COOH groups, and of the hydrophobic sp²-carbon regions, which are close to perfect graphene [18-20]. A lipid bilayer is a self-assembled structure of amphiphilic lipid molecules. Therefore, the
behavior of lipid bilayers on the amphiphilic GO surface is an interesting subject from the viewpoint of interfacial chemistry. In this manuscript, we describe the formation of SLB by vesicle fusion method, and the effect of vesicle size and Ca$^{2+}$ ion.

2. Experiments

2.1. Preparation of graphene oxide
GO was prepared from graphite powder (Z+80, Ito Kokuen Co., Ltd., Japan) by chemical exfoliation according to the modified Hummer’s method [21-23], then the GO suspension was sonicated for 60 min to decrease the size of GO flakes. The GO suspension was dropcast on a thermally oxidized SiO$_2$/Si(100) substrate (87 nm thick SiO$_2$) [24] cleaned by boiling in piranha solution for 10 min followed by sonication in 0.02 M KOH(aq.) for 10 min. The GO/SiO$_2$/Si substrate was observed with an optical microscope and an atomic force microscope (AFM). The width of the GO flakes before sonication was 213 µm at maximum (figure 1a). The size of the GO flakes became 1-10 µm after the sonication. The height of the GO flakes on the SiO$_2$/Si surface obtained from AFM topography was 1.74 ± 0.20 nm (n=40) (figure 1b, and 1c).

![Figure 1.](image)

2.2. Preparation of vesicle suspension and formation of SLB on GO/SiO$_2$/Si substrate
The chloroform solution of dioleoylphosphatidylcholine (DOPC) (Avanti Polar Lipid) and the ethanol solution of fluorescence-labelled lipid (BODIPY-H-PC: Ex/Em = 534/552 nm) (Invitrogen) were mixed at 100:1 molar ratios in a glass vial. The lipid mixture was dried with N$_2$ flow, followed by overnight evacuation. A multilamellar vesicle (MLV) suspension were prepared by suspending the vacuum-dried lipid film into a buffer solution (100 mM KCl, 25 mM HEPES/pH 7.4 NaOH, chemicals were purchased from Wako Pure Chemical Industries, Ltd., Japan) [13]. The MLV suspension was repeatedly frozen and thawed in liquid nitrogen and a water bath at 45 °C, respectively, and was extruded through a 100 nm polycarbonate filter to obtain unilamellar vesicles. The GO/SiO$_2$/Si substrate was incubated in DOPC + BODIPY-H-PC vesicle suspension at 45 °C for 60 min. The excess vesicles in the liquid phase were washed out by exchanging the suspension with the fresh buffer solution.

2.3. Apparatus
An epifluorescence microscope (Olympus IX51) was used in bright-field and fluorescence observation. The GO flakes on the SiO$_2$/Si surface was observed in air. The GO/SiO$_2$/Si surface after the incubation in vesicle suspension was observed in buffer solution with a 60× water-immersion objective lens. We performed fluorescence recovery after photobleaching (FRAP) by irradiating excitation light 225 times brighter than for observation.

In AFM observation (Agilent PicoScan2500), the GO/SiO$_2$/Si surface was observed by using a cantilever with spring constant (C) of 28 N/m (SI-DF40, Seiko Instruments Inc.) in acoustic AC mode in the air. The GO/SiO$_2$/Si surface after the incubation in vesicle suspension was observed by using
magnetically coated cantilever (TYPE I MAC Lever, Agilent, C = 0.6 N/m) in magnetic AC mode in the buffer solution.

3. Results and discussion

3.1. GO/SiO\textsubscript{2} surface incubated in DOPC vesicle suspension

Figure 2a shows the AFM topography of the GO/SiO\textsubscript{2} surface after the incubation in the 100 nm-extruded vesicle suspension. Generally, a full-coverage SLB can be obtained on SiO\textsubscript{2} using 100 nm-extruded vesicle suspensions [12,13]. The GO regions in figure 2a, however, were covered with 13 nm-high protrusions, which were assigned to unruptured vesicles [14]. The transformation from vesicles to SLB did not proceed on GO. From the previous results, it is known that the formation of SLB are assisted if vesicle sizes were decreased by sonication, and/or Ca\textsuperscript{2+} ion was added into buffer solutions [12,13]. Therefore, we studied the effect of the pre-sonication of the vesicle suspension and of the addition of Ca\textsuperscript{2+} ion in the buffer solution on the formation of the DOPC-SLB on the GO/SiO\textsubscript{2} surface (figure 2).

Figure 2b shows the AFM topography of the GO/SiO\textsubscript{2}/Si surface after the incubation in sonicated vesicle suspension. Although a few flat regions were observed on GO, the majority of the GO surface was covered with 16 nm-high protrusion, which were also assigned to the unruptured vesicles. Figure 2c and 2d show the AFM topographies of the GO/SiO\textsubscript{2}/Si surface after the incubation in vesicle suspension containing 5 mM of CaCl\textsubscript{2}. We observed flat morphology on both GO and SiO\textsubscript{2} regions with both the 100 nm-extruded vesicle suspension (figure 2c) and the sonicated vesicle suspension (figure 2d). We found few large protrusions observed in figure 2a and 2b. The surface of each GO flake was flat, the height within each GO flake was constant, and the GO regions were always higher than the SiO\textsubscript{2} regions. These results suggest that SLB was formed on GO. In figure 2c, the height of the GO regions from the SiO\textsubscript{2} regions was 6.86 ± 0.73 nm (n = 40). In figure 2d, the height of the GO regions from the SiO\textsubscript{2} regions were 1.55 ± 0.28 nm (n = 60), or 5.61 ± 0.58 (n = 60).

Figure 3 shows the fluorescence images of the SLB/GO/SiO\textsubscript{2} surface incubated under the same condition as figure 2d. The SLB formed on GO was remarkably darker than that on SiO\textsubscript{2}. This result confirmed that the fluorescence from the BODIPY-H-PC in the DOPC-SLBs was quenched by GO [25-27]. We performed FRAP to study the fluidity of the SLB on GO. After the SiO\textsubscript{2} regions surrounding by GO (figure 3a, dotted circle) was photobleached, the fluorescence at the SiO\textsubscript{2} regions

![Figure 2. AFM topographies of the GO/SiO\textsubscript{2} surfaces after incubated at typical condition, and the cross section profiles at the white line in each AFM topography. (a) Vesicle suspension without sonication in the absence of CaCl\textsubscript{2}. (b) Sonicated vesicle suspension in the absence of CaCl\textsubscript{2}. (c) Vesicle suspension without sonication in the presence of 5 mM CaCl\textsubscript{2}. (d) Sonicated vesicle suspension in the presence of 5 mM CaCl\textsubscript{2}. Image sizes are (a) 50×50 µm\textsuperscript{2}, and (b-d) 10×10 µm\textsuperscript{2}.](image-url)
recovered with time (figure 3b-3d). This result indicates that lipid molecules diffused freely between the SiO$_2$ regions and the surrounding GO regions, therefore that fluid and continuous SLB were formed on GO, as well as on SiO$_2$.

![Figure 3. Fluorescence images of the GO-supported lipid bilayers and FRAP process. (a) Before fluorescence bleaching. (b) 0 s, (c) 180 s, and (d) 600 s after photobleaching. Scale bars correspond to 20 µm.](image)

3.2. Structural model of the SLB/GO system

Figure 4 shows the structural model of GO-SLB system, which we propose based on the height observed in the AFM topography (figure 2d) and the result of FRAP (figure 3). The SLB on the SiO$_2$ regions had fluorescence, and FRAP proceeded. This result indicates that single lipid bilayer was formed on SiO$_2$ similarly to previous studies [13,15]. Single lipid bilayer was also formed on GO, because the height of the GO regions, which was 1.6 nm from SLB/SiO$_2$, was close to the height of GO in figure 1. On the other hand, the GO regions 5.6 nm higher than the SLB/SiO$_2$ was 4.0 nm higher than the single SLB/GO regions. This value corresponded to the thickness of single lipid bilayer observed by AFM. Previous AFM studies show that the thickness of DOPC-SLB observed with tapping mode is 4-5 nm [28-31]. Hence we conclude that two layers of lipid bilayer were formed on GO. Furthermore, the results of FRAP measurement (figure 3) suggests that the lipid bilayers on GO and SiO$_2$ had fluidity and continuity.

![Figure 4. Structural model of the SLB/GO/SiO$_2$ system.](image)
linked with the substrate surface to make a space between the lipid bilayer and substrate [32-35]. The double SLB system on GO may provide another methodology: the first SLB on GO may work as a buffer between the substrate and the extramembrane region of the proteins incorporated in the second lipid membrane, because PC-SLB prevents non-specific adsorption of proteins.

4. Summary
We prepared DOPC-SLB on the GO/SiO₂/Si surface by the vesicle fusion method. Fluid and planar lipid bilayers were formed on GO after the incubation of GO on the thermally oxidized SiO₂/Si substrate in CaCl₂-containing DOPC vesicle suspension. From the AFM observation, we found that not only single lipid bilayer, similar to inorganic substrates, but also double lipid bilayers were formed on GO. We proposed a structural model of the GO-SLB system. This GO-SLB system is the fundamental platform for the measurement of biomolecules in the plasma membrane model using graphene.

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