Oriented Assembly of Purple Membrane on Solid Support, Mediated by Molecular Recognition

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Oriented films of purple membrane patches were assembled on solid supports by the biotin/streptavidin molecular recognition technique. The degree of biotin labeling was 0.58/bacteriorhodopsin. According to the kinetics of absorption change after flash illumination, the photochemical activity of the purple membrane was hardly affected by the biotinylation. The three steps of the oriented assembly were visualized by electron microscopy. According to surface plasmon resonance, about 0.46 ng/mm^2 of streptavidin and 1.16 ng/mm^2 of biotinylated purple membrane were in the assembled films. The amount of the nonspecifically adsorbed purple membrane, without label, was 0.44 ng/mm^2. To test the properties of this oriented purple membrane film, a similar assembly procedure was carried out with the bilayer lipid membrane system. The photoelectric voltage peak of the oriented purple membrane was 157.2 mV, whereas that of nonspecific adsorbed purple membrane was 7.8 mV. The degree of orientation was calculated to be 81% theoretically. Further washing experiments suggested that the nonspecific adsorption may be attributed to both the electrostatic attraction and the hydrophobic interaction.

Introduction

The purple membrane (PM), in the cell membrane of *Halobacterium salinarium*, contains several polar lipids but only one protein, bacteriorodopsin.[1] Bacteriorhodopsin (bR) is an integral membrane protein (MW, 26548) whose seven transmembrane helical segments (A−G) enclose a binding pocket for the all-trans retinal chromophore, bound via a protonated Schiff base to K_{216}.[2] Upon illumination, bR undergoes a photocycle, passing through a series of intermediates, referred to as K, L, M, N, and O. Simultaneously, a single proton is transported through the interhelical channel, from the cytoplasmic to the extracellular medium, and generate a proton gradient that can be used as an energy source for the bacterium.[3]

bR is one of the most promising biomaterials that can be used for energy conversion, optoelectronics, optical storage, information processing, and nonlinear optics. Its utility is based on its unique properties as follows: (1) extraordinary stability against thermal and photochemical degradation,[4] (2) fast photochemical reaction time of less than 5 ps,[5] (3) high quantum yields in the forward and reverse directions,[6] (4) large shift in the absorption spectrum accompanying the photocycle,[7] and (5) the ability to sustain its biological activity when immobilized on solid supports.[8] In bR-based photoelectric devices and other applications, it is essentially important that a nonrandom oriented bR film be formed to generate highly efficient electric response. To achieve a highly oriented film of purple membrane, Langmuir−Blodgett (LB) deposition,[9] electric field sedimentation (EFS),[10] electrostatic layer-by-layer adsorption (LBL),[11] preferential orientation at interfaces,[12] immobilizing matrixes,[13] sol−gel encapsulation,[14] and other techniques have been applied and developed in this research field over the past years. Although these techniques are convenient for operations under many experimental conditions, they do not achieve optimally high degree of orientation, and some questions with regard to the physical mechanisms of orientation and the intrinsic or practical limits to the degree of orientation remain unclear.[15]

Koichi Koyama et al.[16] utilized specific antigen−antibody molecular recognition for bR orientation. Different from techniques such as LB on the base of hydrophobic/hydrophilic interaction and techniques such as LBL and EFS on the base of electrostatic interaction, this specific interaction technique proved to be effective for fabricating highly oriented bR devices. However, it is more involved and less reliable than the other methods because synthesis of antigens and preparations of the monoclonal antibodies and bi-antibodies are necessary.

In this present paper, we report a novel assembly idea that is based on the specific labeling of bR with biotin[17] and the specific molecular recognition between biotin and streptavidin.[18] Streptavidin is a protein that is comprised of four identical subunits, each binding one biotin molecule. The binding affinity between streptavidin and biotin is so high ($k_\alpha = 10^{15}$ M^{-1}) that the formation of this complex can be regarded as nearly irreversible, on a scare nearly comparable to a covalent bond. Therefore, the biotin/streptavidin system has wide practical applications in biological experimentation.[19] In some reports, the biotin/streptavidin technique was applied to assemble DNA[20,21] and some soluble proteins,[22] but the assembly of membrane proteins by this technique had been seldom reported. In our report, streptavidin was first used as a bioreactive-docking matrix to assemble the purple membrane films. The step-by-step assembly is illustrated schematically in Figure 1. By virtue of the specific interaction, the purple membrane can be assembled with nonrandom orientation on supporting films. Because all of the materials are commercial products and the operations are simple and feasible under ordinary experimental conditions, it should

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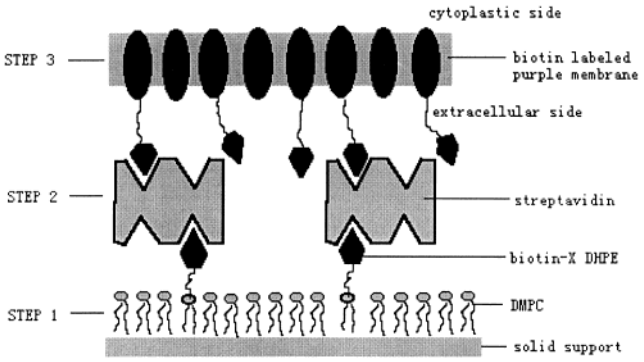

Figure 1. Schematic illustration of the assembly of a triple layer of biotin-lipid/streptavidin/biotinylated purple membrane. Briefly, the biotin-lipid monolayer is first transferred to a solid support by LB technique. Then, the streptavidin is bound to the biotin-lipid as the second layer. Finally, the biotin-labeled purple membrane is coupled to the streptavidin layer.

be possible to apply this method widely in bR-based devices or the potential assembly of other membrane proteins.

Materials and Methods

Reagents. The purple membrane was prepared according to standard procedures[23] and was kept in distilled water at 4 °C. 6-((6-((Biotinoyl)amino)hexanoyl)amino)hexanoic acid, sulfosuccinimidyl ester, sodium salt (biotin-XX, SSE), and *N*-((6-(biotinoyl)amino)hexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (biotin-X DHPE) were purchased from Molecular Probes. 4′-Hydroxyazobenzene-2-carboxylic acid (HABA), biotin *N*-hydroxylsuccinimidyl ester, avidin, streptavidin, lecithin (from soybean), and phosphatidylcholine dimyristoyl (DMPC) were purchased from Sigma. Other reagents are all analytical grade from Fluka and other companies.

Biotin Labeling of Purple Membrane. The biotin labeling procedure of Richard Henderson was followed.[17] In short, 1 mL of a 4 mg/mL suspension of purple membrane was added to a 2 mL reaction tube containing a stir bar and was mixed with 100 μL of 1 M sodium bicarbonate solution (pH 8.5). Subsequently, 100 μL of 10 mg/mL freshly prepared solution of biotin-XX SSE was added to the reaction tube and stirred for 1 h. The sample was then washed 3 times by centrifugation and resuspended in 0.1 M sodium bicarbonate solution (pH 8.5) and left overnight to remove unwanted biotin, which had weakly coupled to hydroxyl groups on the membrane. Finally, the sample was dialyzed against 10 mM PBS buffer (2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) for 2 days at 4 °C. The extent of biotinylation was tested according to the traditional HABA-avidin method.[24]

Spectroscopy Measurements. The characteristic absorption wavelength of both the purple membrane and the biotin-labeled purple membrane was measured on the Hitachi U-3200 spectrophotometer. The flash kinetic absorption changes that reveal the transient accumulation of the intermediate M_{412} was measured on homemade instruments[25] and analyzed by the method of Govindjee et al.[26]

Characterization by Surface Plasmon Resonance (SPR). The characterization of the ligand/receptor binding interaction must be with a highly surface sensitive method. In the present work, the assembly was monitored in real-time by SPR technique.

To prepare gold-coated cover slides, the cover slides were cleaned and hydrophobilized substantially.[27] After being air-dried, a 50-nm gold layer was vapored to the slides under vacuum. A computer controlled LB film balance was used to form the lipid monolayer. The mixture of biotin-X DHPE and DMPC (a molar ratio 1:10) was deposited in small droplets on water surface of a LB film balance. Then the lipid monolayer was compressed to a surface pressure of 40 mM/m and horizontally transferred onto gold-coated cover slides.

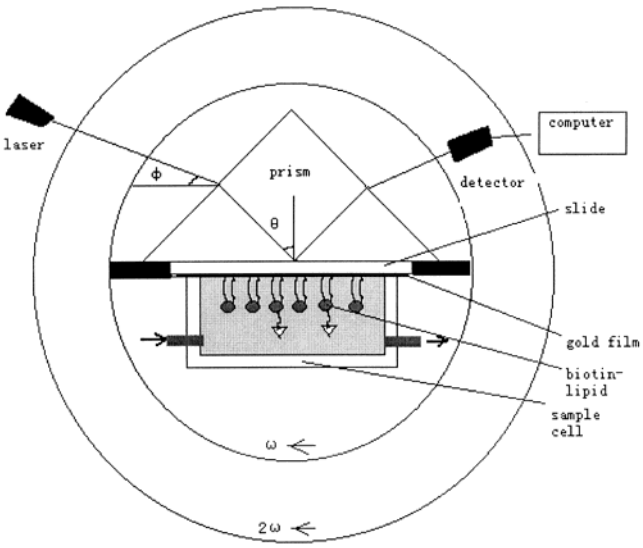

Figure 2. Schematic illustration of the arrangement of the apparatus for the SPR apparatus. Using a semiconductor laser (wavelength of 670 nm), the measurement was performed by varying the incidence angle Φ. The triangular prism (refractive index 1.8) was fitted onto the small rotating stage of this apparatus. As the small rotating stage moves with an angular velocity of ω (0.5 deg/s), the photodiode fitted on the big rotating stage moves at 2ω (1 deg/s). The accuracy of measurements was 0.001°. More details were shown in the report by Sui et al.[27]

The homemade SPR apparatus[27] was shown schematically in Figure 2. After the lipid monolayer was transferred to the gold-coated slide, the slide was carefully adhered, with index matching fluid, to the bottom surface of a triangular prism. Then the sample cell was installed, and the 10mM PBS buffer was slowly pumped into it. After recording the initial resonance angles (Φ_{SPR}) for buffer, the streptavidin (0.05 mg/ml) in 10mM PBS buffer solution was pumped into the cell. After the reaction reach the equilibrium, the cell was washed by PBS buffer to remove nonspecifically adsorbed streptavidin. Subsequently, the solution of the biotin-labeled purple membrane (0.02 mg/mL) was pumped into the sample cell. After the equilibrium, the cell was washed by the same buffer to remove weakly adsorbed biotin-labeled purple membrane. A purple membrane without biotinylation was used in the control experiment to investigate nonspecific adsorption. All of the resonance angles (Φ_{SPR}) were recorded during the whole procedure, and the results were calculated according to Stenberg.[28] The surface mass density (ng/mm^2) of protein binding to the membrane surface has a linear relationship with the change of resonance angle ($\Delta\Phi_{SPR}$), so the 0.1° shift of $\Delta\Phi_{SPR}$ represents the 0.55 ng/mm^2 of protein adsorbed. The number density of protein molecules (N) can also be calculated by the equation: N (number/mm^2) $= 6.023 \times 10^{15}(0.55)\Delta\Phi_{SPR}/MW$.

Characterization by Electron Microscope (EM). To visualize the each step of the assembly, the EM technique was used. First, 17.5 μL of PBS buffer (10 mM, pH 7.2) was place in a Teflon well (4 mm in diameter and 0.5 mm in depth), and 1 μL of the sample prepared by mixing biotin-X DHPE and DMPC (a molar ratio 1:10, total concentration 1 mg/mL) in the mixture of chloroform/methanol (a molar ratio 3:1) was spread at the

TABLE 1: Comparison of the Characteristic Absorption Wavelength and Lifetimes of M_f and M_s for the Purple Membrane and Biotinylated Purple Membrane[a]

	degree of biotinylation (biotin:bR)	characteristic absorption wavelength (nm)	lifetime of M_f (ms)	lifetime of M_s (ms)
purple membrane		568.0 ± 0.5	3.5 ± 0.4	8.5 ± 0.8
biotin-labeled purple membrane	0.58 ± 0.10	567.0 ± 0.5	3.4 ± 0.5	8.2 ± 0.6

[a] The degree of biotinylation was also determined.

interface. The lipid monolayer was then picked up on hydrophobic carbon-coated EM grids and negatively stained with 1% uranyl acetate for 60 s. The sample was examined by PHILIPS TECNAI 20 transmission electron microscope to characterize the first step of the assembly. Second, the mixture of lipids was spread on the streptavidin solution (0.05 mg/mL) and incubated for 30 min. Then this biotin-lipid/streptavidin layer was picked up with carbon-coated EM grids, prepared and examined as described previously to characterize the second step of the assembly. Third, the biotin-lipid/streptavidin layer prepared in the second step was transferred to carbon-coated EM grids, and then the grids were placed on a 0.02 mg/mL suspension of biotin-labeled purple membrane to incubate for 60 min. After the negative stain, the third step of the assembly was characterized.

Characterization by Photoelectric Response. The apparatus for measuring the photovoltage signals of assembled purple membranes in the bilayer lipid membrane system (BLM) was homemade according to Tien.[29]

A 100-W tungsten lamp with heat protection filters was used as a light source. The calomel electrodes were protected from illumination to avoid photo effects at the electrode interfaces. The electrolyte was 100 mM KCl and 5 mM $CaCl_2$ (pH 6.4, unbuffered). The membrane-forming solution was a mixture of 1 mg of biotin-X DHPE and 10 mg of lecithin in 200 μL of decane. The planar bilayer lipid membrane was formed by "painting" the forming solution over an aperture (150−200 μm in diameter) in a thin Teflon foil that separates the cell into compartment A and compartment B.[30]

When the mixed bilayer was formed and stable (generally after 30 min), the streptavidin solution (0.05 mg/mL), which had been dialyzed against electrolyte for 24 h, was pumped into the compartment B. Thirty minutes later, the streptavidin was washed away by electrolyte and the biotin-labeled purple membrane (0.02 mg/mL, also dialyzed against electrolyte) was pumped into the compartment B. The photovoltage signals were recorded in real time when the purple membrane was adsorbed to the bilayer. In the control experiment, the purple membrane (0.02 mg/mL) without biotin labeling was adsorbed to the bilayer and the photovoltage was recorded.

All of the operations were carried out at the same room temperature except where otherwise indicated.

Results and Discussion

This is a report on the first application of the biotin/streptavidin technique for the fabrication of a bR film. According to its characterization by EM, SPR, and photoelectric response, an oriented assembly of the purple membrane on biotin-lipid/streptavidin layers was achieved.

Biotin Labeling. In present work, biotin-XX SSE, was used to label the purple membrane. Theoretically, the long hydrophilic spacer (13.35 Å approximately in length) allowed the biotin moiety to protrude above the surface of the purple membrane and to interact with streptavidin fully, because the lysine residues that had been labeled with biotin were located on the hydrophilic surface of the purple membrane. In a control experiment, we even used biotin *N*-hydroxyl succinimidyl ester (without spacer) to label the purple membrane. The amount of the biotinylated purple membrane, which had assembled to streptavidin layer, was not distinctly reduced (data are not shown). We chose biotin-XX SSE for labeling to ensure the optimal assembly of biotinylated purple membrane to streptavidin layer.

For the oriented assembly, uniform and selective biotin labeling of the purple membrane must be achieved (as illustrated in Figure 1). If both sides of the membrane are labeled, the assembly of the biotin-labeled purple membrane on the streptavidin layer will be random. Although the lysine residues are located on both surfaces, Henderson[17] has confirmed that under alkaline conditions (pH 8.5−9.0) only one lysine residue, K129 on the extracellular surface, can be labeled by biotin derivatives. This important result makes the idea of the oriented assembly of biotinylated purple membrane on streptavidin feasible.

We labeled the purple membrane according to Henderson's method. The stoichiometry of biotinylation was 0.58 ± 0.10 mol/mol (Table 1, calculations in detail, according to Green's method[24] not shown here) in our experiments. This indicated that there was a biotin label on about one of every two bR molecules in the purple membrane (there are about 18 000 bR molecules in one patch of the purple membrane[31]).

Previous research seldom reported whether the biotinylation could affect the photochemical activity of bR. To characterize the effect of biotin labeling on the activity of bR, UV−vis absorption spectra and flash kinetic spectra were recorded and analyzed. After the biotinylation, the characteristic absorption wavelength of the purple membrane had hardly shifted, from 568 to 567 nm (Table 1). This suggested that the conformation of bR had not been perturbed. According to the flash kinetic spectra of the intermediate M_{412} (shown in Figure 3, parts a and b), the lifetimes of M_s (the slow component of M_{412}) and M_f (the fast component of M_{412}) for the purple membrane were 3.5 and 8.5 ms, respectively. The lifetimes of M_s and M_f for the biotin-labeled purple membrane were 3.4 and 8.2 ms, respectively (Table 1). Thus, the photocycle of bR had been hardly affected by the biotin labeling, and the biotin-labeled purple membrane maintained its photochemical activity. Only under this precondition would the assembly of biotinylated purple membrane be significant and feasible.

Assembly Procedure. The assembly process of the biotinylated purple membrane was characterized by both SPR and EM techniques. The SPR data discussed here were in the surface mass density (ng/mm^2) form, which had been transformed from the original $\Delta\Phi_{SPR}$ (°) form (Figure 4).

To obtain an optimal biotin-lipid/streptavidin interaction, the biotin-X DHPE, which has a long hydrophilic spacer (about 6.91 Å in length), was mixed with DMPC (molar ratio 1:10) to form the monolayer. It had been reported that streptavidin could not bind tightly to a pure biotin lipid because of the steric hindrance caused by densely packed biotin molecules.[27,32] This steric hindrance could be overcome by "diluting" the biotin functionalities with an unfunctionalized lipid. In our experi-

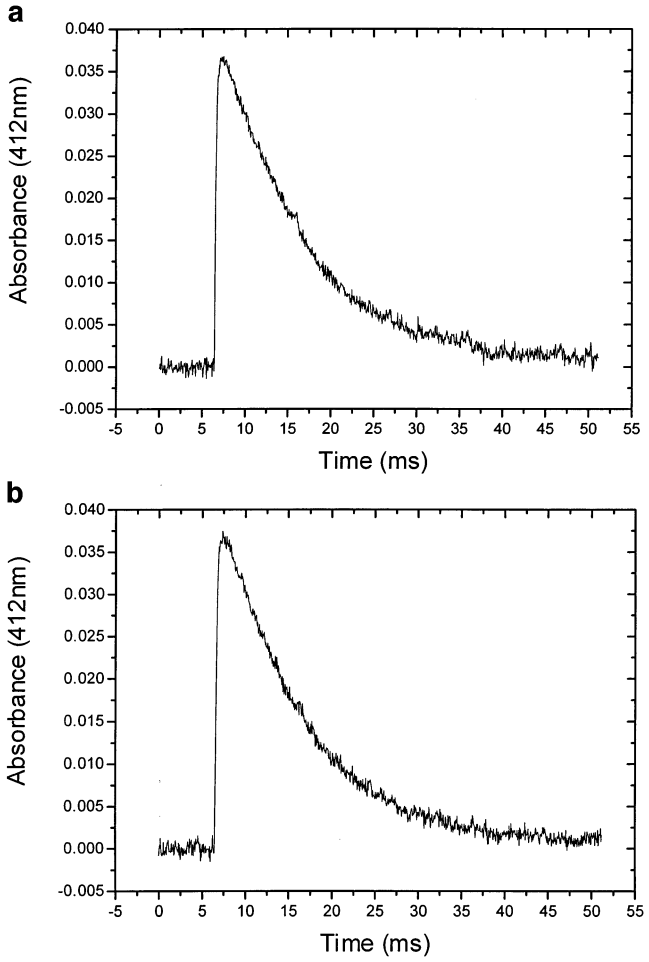

Figure 3. (a) Flash kinetic spectrum of the purple membrane at 412 nm. (b) The flash kinetic spectrum of biotinylated purple membrane at 412 nm. The concentrations of both samples were 10 μM, which were suspended in the PBS buffer (2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 150 mM NaCl, pH 7.2).

ments, we used DMPC as the unfunctionalized lipid and biotin-X DHPE as biotin functionalized lipid. To achieve an optimal assembly of streptavidin on biotin lipid layer, the molar ratio (DMPC: biotin-X DHPE) was chosen as 10:1, based on the studies of Sui.[27]

Then, the monolayer of the mixed lipid was formed on solid support (Figure 5a) by LB technique, and streptavidin was adsorbed and arrayed densely on this lipid layer (Figure 5b). It was difficult to estimate the number of streptavidin molecules that had been adsorbed specifically from the EM images. However, the SPR observations (Figure 4a) clearly revealed that the surface mass density of streptavidin that had been adsorbed on the lipid supports was around 0.46 ng/mm^2 (equal to 4.61 $\times$ 10^{10} streptavidin molecules per square millimeter). In the work of Darst et al.,[33] the "H" shaped domains of streptavidin were observed under specific conditions because of the formation of 2-D crystal, whereas they were absent in our experiments. However, it was not necessary for streptavidin to form 2-D crystals in our assembly method. It is known that the diameter of the purple membrane is about 0.5 μm and the area of one piece of purple membrane is therefore 0.196 μm^2 approximately. There are around 18 000 bR molecules in one patch,[31] so the concentration of bR is 9.18 $\times$ 10^{10} molecules per square millimeter. Because the streptavidin concentration was 4.61 $\times$ 10^{10} streptavidin molecules per square millimeter on biotin lipid supports, the ratio of assembled streptavidin to bR molecules

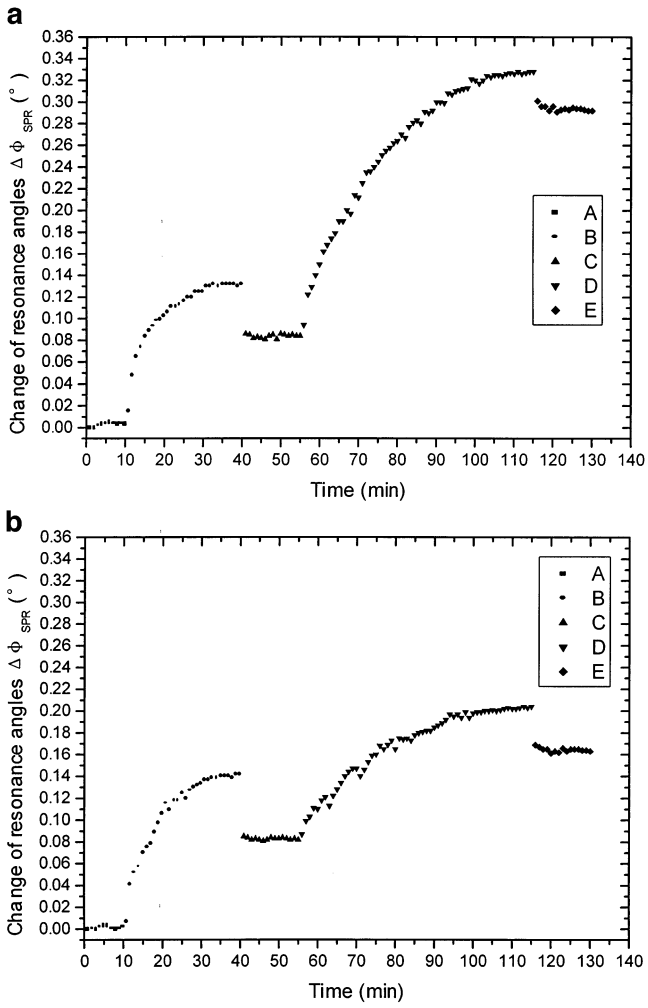

Figure 4. (a) Change of resonance angles $\Delta\Phi_{SPR}$ during the assembly procedure. A: The change of resonance angles $\Delta\Phi_{SPR}$ when 10 mM PBS buffer (2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) was pumped into the sample cell. B: The change of resonance angles $\Delta\Phi_{SPR}$ when streptavidin (0.05 mg/mL) in 10 mM PBS buffer solution was pumped into the cell. C: The change of resonance angles $\Delta\Phi_{SPR}$ when 10 mM PBS buffer was pumped. D: The change of resonance angles $\Delta\Phi_{SPR}$ when biotin-labeled purple membrane (0.02 mg/mL) in 10 mM PBS buffer was pumped. E: The change of resonance angles $\Delta\Phi_{SPR}$ when 10 mM PBS buffer was pumped. (b) The change of resonance angles $\Delta\Phi_{SPR}$ during the control experiments procedure. A, B, C, and E were identical to Figure 4a's. D: The change of resonance angles $\Delta\Phi_{SPR}$ when unlabeled purple membrane was pumped into the cell.

is around 1:2. Thus, the surface mass density of streptavidin (0.46 ng/mm^2) was high enough to induce the oriented assembly of the biotin-labeled purple membrane.

In virtue of the extremely high binding force between biotin and streptavidin ($k_\alpha = 10^{15}$ M^{-1}), the biotin-labeled extracellular surface of the purple membrane could be firmly adsorbed to the streptavidin layer. The SPR curves (Figure 4a) showed that 1.16 ng/mm^2 of biotinylated purple membrane was adsorbed, and the EM photos (Figure 5c) showed that the labeled purple membrane was arrayed densely on the streptavidin layer. We also observed the overlap among adjacent fragments of the purple membrane. With the diameter of purple membrane being 0.5 μm, which is much larger than that of soluble proteins, it is reasonable that the overlap could occur among membrane fragments on a plane support surface.

Photoelectric Response Properties. The basic physiological function of bR in the purple membrane is to transfer the proton

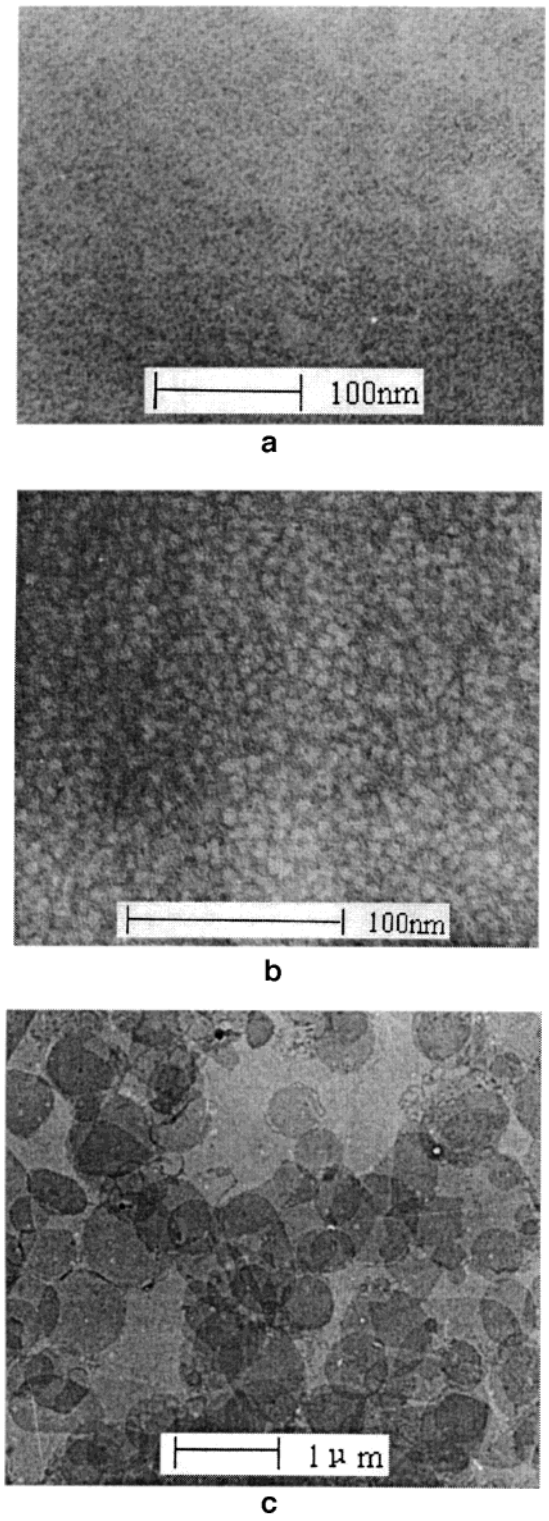

Figure 5. Three steps of the oriented assembly procedure were characterized by EM. (a) The lipid monolayer of mixed biotin-X DHPE and DMPC. (b) The streptavidin layer. (c) The biotinylated purple membrane.

across the membrane under illumination. The main functional property of purple membranes is therefore the photoelectric response. We assembled the biotin-labeled purple membrane on the BLM system in order to test the photoelectric response signal of this oriented film.

After the biotin-labeled purple membrane had been adsorbed onto the biotin-lipid/streptavidin bilayer and this adsorption reaction had approached equilibrium, the photovoltage signal

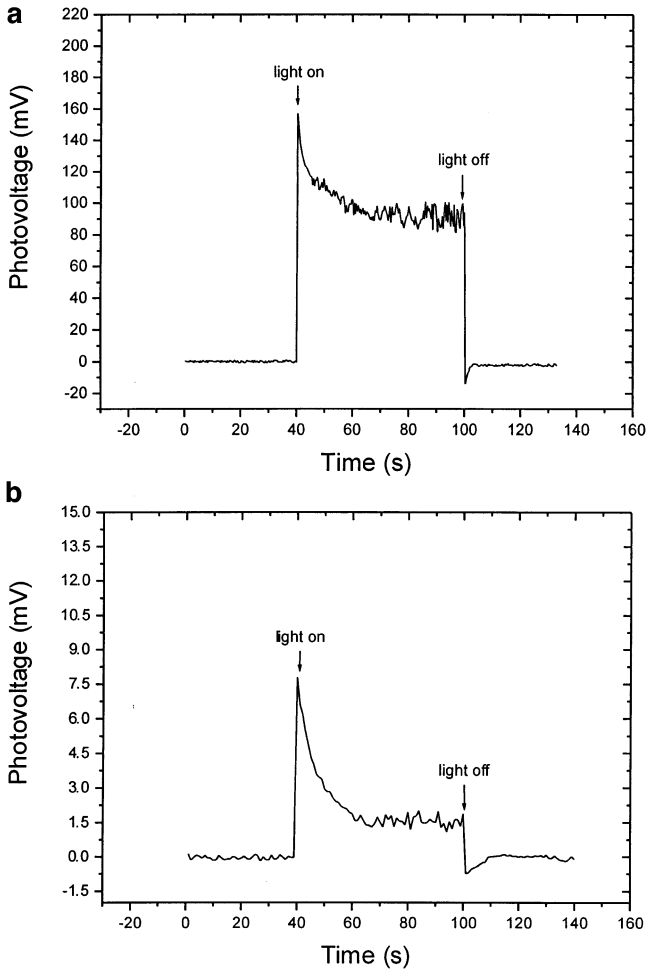

Figure 6. (a) Photovoltage signal of the biotin-labeled purple membrane assembled onto the bilayer lipid membrane system. (b) The photovoltage signal of the control sample (without biotinylation).

was recorded and shown in Figure 6a. When this orientedly assembled purple membrane was illuminated, the photovoltage peak value reached 157.2 mV. In the control experiment (Figure 6b), the photovoltage peak value of nonspecific adsorbed purple membrane was only 7.8 mV. The strong photovoltage signal generated by the biotin-labeled purple membrane, as compared to the control experiment, showed that the biotin-labeled purple membrane was successfully oriented by the biotin/streptavidin interaction.[34] The circuit analysis had also demonstrated that the positive polarity of photovoltage signal, representing transport of the proton, was in the direction from compartment B to compartment A. This meant that most of the biotin-labeled purple membrane in compartment B had been adsorbed specifically onto the biotin-lipid/streptavidin bilayer with extracellular side, where K_{129} is located. Another important result was that the polarity of the photovoltage signal of the nonspecific adsorbed purple membrane without biotinylation was the same as that of the oriented purple membrane with biotinylation. Therefore, we may conclude that the amount of the purple membrane deposited at random with the cytoplasmic side facing the streptavidin and the bilayer is smaller than that deposited at random with extracellular side facing the streptavidin and the bilayer.

We recorded the photovoltage signal of the assembled purple membrane in real time also. Figure 7 showed that, as the purple membrane was deposited onto the bilayer, the photovoltage signal increased and approached equilibrium. The photovoltage signal of the biotin-labeled purple membrane reached the

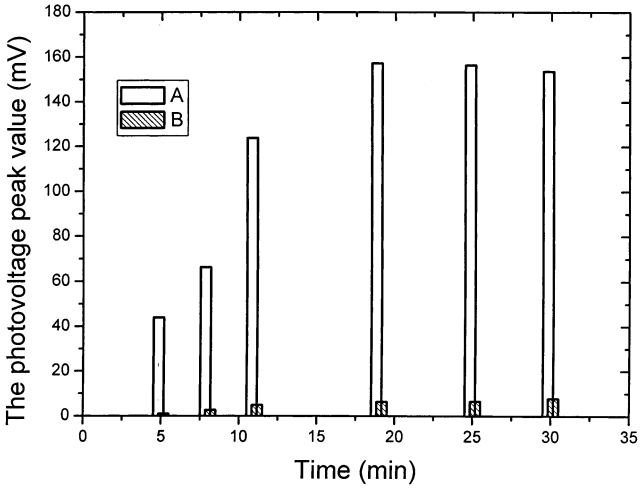

Figure 7. Changes of the photovoltage peak values as the purple membrane was assembled to the bilayer. A: The biotin-labeled purple membrane. B: The purple membrane (control).

maximum after an assembly time of about 19 min. However, the photovoltage signal of the control sample reached the maximum only when after 30 min. Therefore, assembly of the biotin-labeled purple membrane was faster. This indicated that the specific adsorption taking place at the interface was faster than the nonspecific adsorption. The same conclusion can also been drawn from the SPR results.

Nonspecific Adsorption. In the report of Koyama et al., the degree of orientation was about 85% when the purple membrane was deposited with the extracellular side facing the supports.[16] Theoretically, the degree may reach 100% by the antigen−antibody specific interaction, but the authors neither explained the reason about 15% of purple membrane was still adsorbed with cytoplasmic side facing the supports nor gave any suggestion to improve the degree of orientation. In our experiments also, the nonspecific adsorption was observed.

To investigate the amount and the properties of nonspecific adsorption, it was desirable to get the quantitative data. By SPR characterization, the amount of nonspecific adsorption of the purple membrane could be precisely obtained. In the control experiments (Figure 4b), all of the operations were identical to the oriented assembly's (Figure 4a), except that the biotin-labeled purple membrane was replaced with unlabeled purple membrane. This adsorption was defined as nonspecific adsorption. The surface mass density of purple membrane was now lower, about 0.66 ng/mm^2. After 0.22 ng/mm^2 of purple membrane was washed off by PBS buffer, the final density was 0.44 ng/mm^2.

Generally, the photoelectric response is used to characterize whether an oriented purple membrane is well formed or not.[34] However, only the relative changes of photoelectric signals generated by purple membranes with different degrees of orientation can be tested and the absolute values of the degrees of orientation cannot be obtained by this method. Koyama et al. first combined the EM technique with monoclonal antibody techniques to characterize the degree of orientation. As illustrated in the Introduction, we did not intend to use this complicated technique. Instead, we first attempt to use SPR technique to calculate the degree of orientation based on some simple assumptions. Once the total amount of the adsorption and the amount of nonspecific adsorption of the purple membrane were obtained, the degree of orientation could be calculated. We assumed, first, that the nonspecifically adsorbed purple membrane was deposited in a random manner,[35] whereas that of specific adsorption was done in a nonrandom manner; that is, the degrees of orientation were expected to be 50% for the former and to be 100% for the latter. In our experiments, the total amount of the assembled biotinylated purple membrane was 1.16 ng/mm^2, and the amount of nonspecific adsorption was 0.44 ng/mm^2. Therefore, the amount of labeled purple membrane that was adsorbed specifically was 0.72 ng/mm^2. From the assumption that half of the nonspecifically adsorbed purple membrane faces streptavidin with its extracellular surface, the total amount of purple membrane whose extracellular surfaces faced the streptavidin layer was 0.94ng/mm^2. Therefore, we can conclude that the degree of orientation of purple membrane was at least 81%.

In the calculation above, we substituted the amount of the nonspecific adsorbed, nonlabeled purple membrane that was obtained from the control experiment (Figure 4b) for the amount of the nonspecific adsorbed, biotin-labeled purple membrane, because the actual amount of the nonspecific adsorbed, biotin-labeled purple membrane could not be obtained from the SPR data in Figure 4a. In fact, the amount of the former was more than that of the latter. First, there were an estimated 18 000/2 = 9000 biotin molecules labeled on the extracellular side of the purple membranes, and there seemed to be no other option for a membrane with its extracellular side toward the streptavidin layer than the specific binding adsorption. Second, the photoelectric response and SPR data showed that the specific adsorption taking place at the interface between streptavidin and biotin-labeled purple membrane was faster than the nonspecific adsorption. Because of this dominant specific adsorption, the surface area accessible to the nonspecific adsorption was expected to be reduced. Third, the photovoltage data (Figure 6b) suggested that even the deposited purple membrane at random had a certain degree of orientation, with the amount of the purple membrane with the extracellular side facing the interface larger than that with cytoplasmic side. Therefore, we concluded that the actual value of the degree of orientation might be greater than the calculated 81%.

Leckband analyzed the influence of protein and interfacial structure on the self-assembly of orientated protein arrays.[36] He indicated that the nonspecific adsorption was dominated by an electrostatic attractive force, which was determined, not by the net protein charge, but by the local electrostatic details of the interacting protein surface. Because some positively charged amino acid residues were located on both surfaces of the purple membrane,[37] the nonspecific electrostatic attractive forces between purple membrane and streptavidin (negatively charged at pH 7.2[36]) were considered reasonable. To analyze the interfacial properties of purple membrane, here we tried to reduce the nonspecific adsorption by washing with different buffers. When the general PBS buffer (2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) was used in control experiments (Figures 4b and 8), the final surface mass density of unlabeled purple membrane was 0.44 ng/mm^2 with only 33% of purple membrane washed off. When the buffer with much higher ionic strength (2 M NaCl, 2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, pH 7.2) was used, the results were almost the same (Figure 8). In a report by Sui et al.,[27] the buffer containing 5 mM Mg^{2+} had been proved effective to remove the nonspecific adsorption of soluble proteins. When we used this buffer with the concentration of $MgCl_2$ up to 100 mM (2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 150 mM NaCl, pH 7.2), the final density of nonspecifically adsorbed purple membrane changed little (Figure 8). These washing experiments suggested that, besides the nonspecific electrostatic force, other forces could result in the

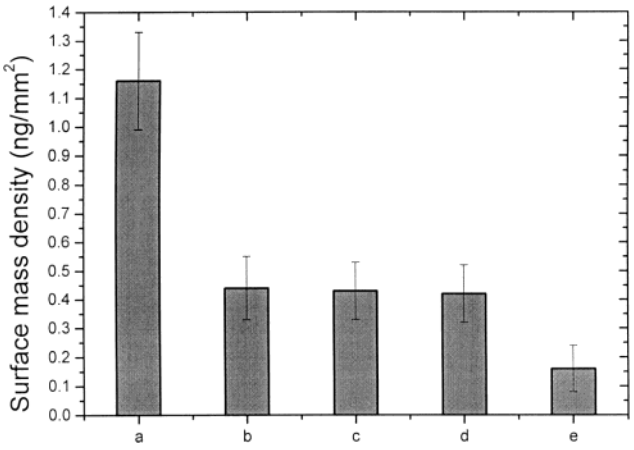

Figure 8. Comparison of the amounts of the adsorbed purple membrane after washed with different buffers. a: The surface mass density of biotin-labeled purple membrane after washed with 10 mM PBS buffer (2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 150 mM NaCl, pH 7.2). b, c, d, and e: The surface mass density of unlabeled purple membrane after washed with 10 mM PBS buffer, 2 M NaCl buffer (2 M NaCl, 2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, pH 7.2), 100 mM $MgCl_2$ buffer (100 mM $MgCl_2$, 2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 150 mM NaCl, pH 7.2), and 30 μM TWEEN 20 buffer (30 μM TWEEN 20, 2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 150 mM NaCl, pH 7.2), respectively.

nonspecific adsorption. We tried a buffer of TWEEN 20 (30 μM, pH 7.2, 2.7 mM NaH_2PO_4, 7.3 mM Na_2HPO_4, 150 mM NaCl), a mild nonionic surfactant.[38] Under its CMC (critical micelle concentration 59 μM), TWEEN 20 would interact with the surface of purple membrane via hydrophobic interaction force without solubilizing this membrane protein.[39] After washed with this buffer, the remainder was only 0.16 ng/mm^2. Therefore, we concluded that TWEEN 20 molecules had interacted with the local hydrophobic surfaces of the purple membrane and competitively reduced the hydrophobic interaction between the purple membrane and streptavidin. That resulted in the obvious desorption of the nonspecific adsorbed purple membrane from streptavidin. Considering that the TWEEN 20 buffer may inhibit the specific interaction of biotin moiety with streptavidin, we also monitored the assembly procedure of the biotinylated purple membrane in this buffer system with SPR technique. Neither obvious reduced surface mass concentration of streptavidin on biotin lipid layer nor that of biotinylated purple membrane on streptavidin layer was observed in control experiments (data not shown). These distinct results demonstrated that the nonspecific adsorption was partially due to the hydrophobic interaction between the surfaces of both purple membrane and streptavidin.

In the assembly of soluble proteins, the nonspecific adsorption, which was generally resulted from the electrostatic attractive force, could be eliminated by buffers' washing.[27] However, in the case of membrane proteins, the results seemed different. Considering that bR is a transmembrane protein and the purple membrane is a patch of 2-D crystal lattice that contains both bR and several lipids, we believe that the surface properties of the purple membrane are more complicated than soluble proteins. To eliminate nonspecific adsorption and thereby improve the degree of orientation, the optimal buffers, which can reduce both the electrostatic attractive force and the hydrophobic interaction force, will be studied intensively. Future research of the oriented assembly of purple membrane by molecular recognition should focus on the surface properties of both streptavidin and purple membrane.

Conclusion

A triple layer of the biotin-lipid/streptavidin/biotinylated purple membrane was assembled by specific molecular recognition. The photochemical activity of the purple membrane was hardly affected by biotin labeling. EM visualized the assembly procedure, and SPR showed precise data of streptavidin and purple membrane adsorbed. The photoelectric response data further indicated that this orientedly assembled purple membrane could generate a remarkably large photovoltage signal, much larger than the signal of purple membrane deposited at random. The final degree of orientation of the purple membrane was approximately 81%, which was close to the earlier results with antibody-aided orientation (85%). Further experiments suggested that the nonspecific adsorption of purple membrane might be due to both the electrostatic attraction and the hydrophobic interaction.

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