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A Planar Bilayer Lipid Membrane Sensor Using a Miniaturized Auto-patch System

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A bilayer lipid membrane sensor was constructed with a miniaturized auto-patch system. The performance of the patch system was optimized to obtain an analytically relevant signal. A biosensor based on an anti-BSA-antibody as a receptor and gramicidin as a signal transduction element was demonstrated. The sensor for BSA exhibited a detection limit of pg/mL level for BSA.

Keywords Bilayer membrane sensor, miniaturized auto-patch system, anti-BSA antibody, BSA

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Introduction

Free-standing planar bilayer membranes can be formed by traditional methods, such as painting,[1] monolayer folding[2,3] and tip-dip[4] methods. These membranes have long been used in the field of electrophysiology for studying isolated and reconstituted proteins,[5] and also in the development of biosensors.[6,7] More recently, mechanically stable bilayers have been be exploited, which can be formed, often semi-automatically, by virtue of advances in the bilayer formation technique.[8,9] Therefore, the design of transmembrane signaling associated with stable planar bilayer membranes will be interesting for constructing sophisticated biosensors that respond to a variety of targets.

In the present paper, we describe the construction of a biosensor with a miniaturized auto-patch system. The modernized bilayer formation method was pioneered by Schmidt *et al.*[10] in 2000 on an aperture in a SiO_2/Si_3N_4 septum and later by Fertig *et al.*[11,12] in 2001 on an aperture in a glass septum. The formation of a bilayer lipid membrane is automatically achieved by rupturing giant unilamellar vesicles on the small aperture (diameter, 1 – 2 μm) of a glass slip.[10-12] However, the application of such a system for the development of a bilayer lipid membrane sensor has not been exploited yet, seemingly because optimization of the patch system is necessary to be optimized as a biosensor. Especially, the introduction of molecular-recognition sites, together with signal transduction ability, is essential to obtain a molecular sensing system. We demonstrate a bilayer lipid membrane sensor for bovine serum albumin (BSA) based on a membrane-bound receptor approach using gramicidin channels. The ion-transport properties of gramicidin A channels have been well establisehed,[13-17] and hence the use of gramicidin A as a signal transduction element will be interesting.

In our previous papers, we showed that the fraction of channel opening with a short lifetime is useful as an analytical signal for the membrane bound receptor approach.[18,19] Later we extended to the case that an integrated channel current is used as an analytical signal, rather than the channel opening fraction.[20] This biosensor based on an integrated current is more sensitive because of the amplified feature of an ion-flux through the channel. This relied on tuning of the membrane composition to obtain channel events with a long lifetime. In this paper, taking it into consideration, the integrated channel current approach, a bilayer lipid membrane sensor is constructed based on the multi-channel activity of gramicidin A. The magnitude of integrated currents for the multi-channel case is much larger than the single-channel case, though only the relative or normalized currents have to be used as an analytical signal. The combined use of the integrated multi-channel current approach and a mechanically stable bilayer may lead to the development of a practical bilayer-based sensor with high sensitivity.

Materials and Methods

Reagents

Gramicidin A and albumin from bovine serum (BSA, >97%) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, 10 mg/mL chloroform solution), 1-2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 10 mg/mL chloroform solution) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol (Chol), sorbitol and *N*-hydroxysuccinimide (NHS) were obtained from Wako Pure Chemicals Co. (Osaka, Japan). Cholesterol was recrystallized three times from methanol. 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) and 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC) were obtained from Dojindo Laboratories (Kumamoto, Japan). Rabbit polyclonal anti-BSA antibody

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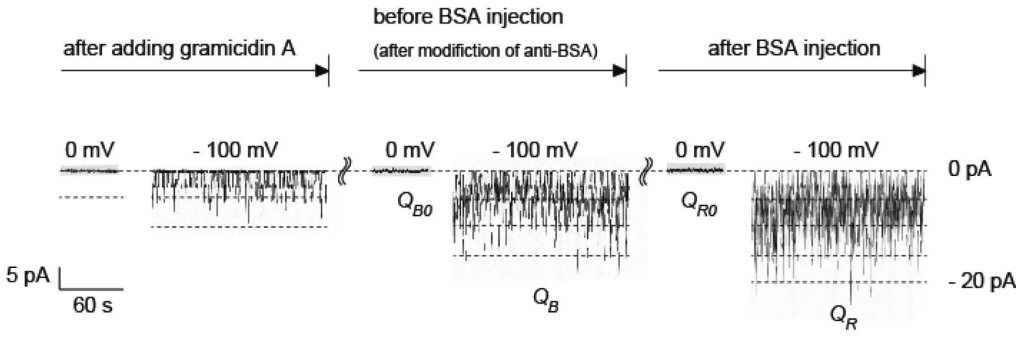

Fig. 1 Response of a bilayer lipid membrane sensor and an illustration for analyzing an integrated current. The response (R) of a bilayer membrane sensor was evaluated according to the following steps. At first, currents were recorded at 0 mV for 1 min and at –100 mV for 3 min. After modification of anti-BSA antibody, currents were recorded again at 0 mV for 1 min. After 30 s, current measurements were started at –100 mV for 3 min to obtain Q_{B0} and Q_B respectively. Then, currents were measured again in a similar manner as in trace (b) after the addition of BSA to obtain Q_{R0} and Q_R respectively.

(anti-BSA) was obtained from Funakoshi (Tokyo, Japan). Other reagents used were all of analytical reagent grade. Milli-Q water (Millipore reagent water system, Bedford, MA) was used throughout the experiments.

Preparation of giant unilamellar vesicles

Giant unilamellar vesicles (GUVs) were prepared from a lipid mixture consisting of DPhPC, DOPE and cholesterol in chloroform at a molar ratio of 4:0.05:1 by electroformation[21-23] using Vesicle Prep Pro (VPP) (Nanion Technlogies, GmbH, Germany). A 20-μL portion of the lipid mixture in chloroform was put on an indium tin oxide (ITO)-coated slide glass and air-dried to a lipid film. After setting a silicon O-ring on it, 0.30 mL of 1 mol/L sorbitol in Milli-Q water was added. Then, an electric field (5 Hz, ac 3 mV) was applied through two ITO-coated slide glasses for 2 h. The GUV suspension was recovered and stored in a microcentrifuge tube (1.5 mL).

Formation of a bilayer lipid membrane

Planar lipid bilayer membranes were formed with an autopatch-clamp apparatus (Port-a-patch, Nanion Technologies GmbH, Germany) and a sensor chip (pore size, ~1 μm) having a resistance of 3 – 5 or 8 – 12 MΩ. Since a horizontal planar bilayer membrane is formed, the upper side of the horizontal bilayer membrane is called the *cis* side, and the bottom side as the *trans* side for convenience. A 10-μL portion of a 0.15 mol/L KCl solution containing 10 mM CH_3COOH/CH_3COOK (pH 4.0) (abbreviated as an acetate solution) was mounted in a *trans* chamber, and an acetate buffer solution was run in a *cis* chamber. Then, a 5-μL of GUV suspension was added to a *cis* chamber (~120 μL) for bursting GUVs under a negative pressure of 20 – 30 mbar. Any excess of GUVs was removed by running an acetate solution with a flow rate of 0.50 mL/min.

Measurements of channel currents

Current recordings were performed at an applied potential with an EPC-7 amplifier (List-Electronics, Co., Darmstadt, Germany) with an 8-pole low-pass filter (NF-Electronic Instruments, Kanagawa, Japan). Currents were stored on-line using a PHYSICO PC computer (PHYISICO-Tech Ltd., Tokyo, Japan) in which a pCLAMP software Ver. 8.1 (Axon Instruments Inc., Burlingame, CA) was installed. All measurements were carried out at room temperature. The magnitude of the channel currents at –100 mV were evaluated by using Fechan (pClamp 8.1, AD instruments Inc.,) according to

$$Q = \int_0^t i\,dt. \tag{1}$$

Since the number of gramicidin channels in the lipid bilayer membranes varied from one membrane to another, the response (R) was normalized according

$$R = \frac{Q_R - Q_{R0}}{Q_B - Q_{B0}}, \tag{2}$$

where Q_R is the magnitude of an integrated current at –100 mV for 1 min after the injection of BSA, Q_{R0} is that recorded at 0 mV for 1 min after the injection of BSA, Q_B is that at –100 mV for 1 min before BSA injection, and Q_{B0} is that at 0 mV for 1 min before BSA injection (Fig. 1).

The probability of observing multi-channel responses was compared between sensor tips having different resistance, *i.e.*, different pore sizes. There was no significant difference between the resistance of 8 – 12 MΩ (n = 150) and the resistance of 3 – 5 MΩ (n = 127) for multi-channel recordings.

Modification of receptor to a lipid bilayer membrane

After channel currents due to gramicidin A were observed, an acetate buffer solution (pH 4.0) containing 10% methanol was run in a *cis* side chamber in order to wash off any excess gramicidin A, followed by running an acetate buffer solution. Then, a 10 μL-portion of anti-BSA antibody (10^{-2} mg/mL, anti-BSA) in an acetate buffer solution was added to a *cis* chamber, followed by the addition of 20 μL of a mixture of 0.50 mol/L NHS and 0.06 mol/L EDC in an acetate buffer solution. After incubation for 15 min, an acetate buffer solution was run for 10 min at a flow rate of 0.50 mL/min.

Impedance analysis

Electrochemical impedance spectroscopy for planar bilayer membranes formed by the monolayer holding method was performed in a 4-electrode system with a high-resolution impedance spectrometer (INPHAZE, Sydney, Austria), as described in our previous paper.[20] The electrochemical cell contained four Ag-AgCl electrodes. A 10-mV amplitude sine-wave signal perturbation was applied in the 0.1 – 10^6 Hz frequency range. All experiments were performed at room temperature. The high-frequency capacitance is dominated by a bulk solution rather than a lipid bilayer. The low-frequency capacitance (1 Hz) is related to the thickness, d, of the bilayer by

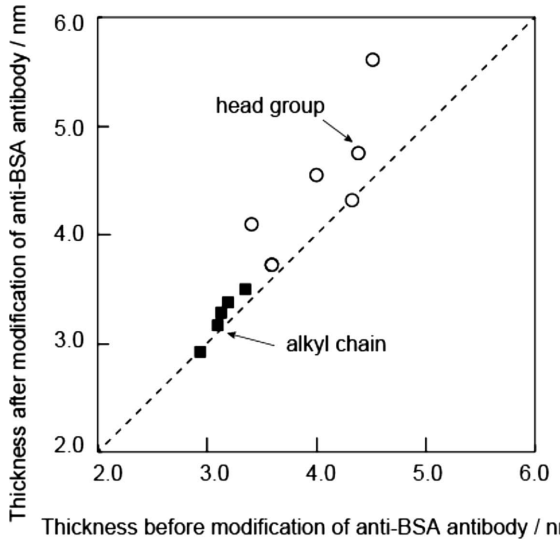

Fig. 2 Change in the membrane thickness caused by a modification of the anti-BSA antibody in 150 mM KCl containing HEPES/KOH at pH 7.4 by electronic impedance spectrometry. The membrane area was assumed to be equal to the geometrical area of an aperture (~200 μm) of a Teflon film. Hydrophobic thickness of carbon chain regions were calculated from specific capacitance at 1 Hz using $\varepsilon_0 = 8.85 \times 10^{-12}$ and $\varepsilon_1 = 2.1$. Thickness of head group regions were calculated from specific capacitance at 1 Hz using a dielectric constant of $\varepsilon_2 = 30$.

$$d = \frac{\varepsilon_0 \varepsilon_1}{C_m} \quad (3)$$

where ε_1 is the dielectric constant of the alkyl chains (2.1) or the head groups[24] (30); ε_0 is the permittivity of free space (8.85×10^{-12}) and C_m the capacitance of a lipid bilayer per unit area. The bilayer area was assumed to be equal to the aperture area of a Teflon film.

Results and Discussion

Modification of receptor on a bilayer lipid membrane

The introduction of a receptor on a bilayer lipid membrane was based on the membrane bound receptor approach, *i.e.*, the receptor is linked to lipid molecules in a lipid bilayer membrane. An anti-BSA antibody was bound to DOPE by the amine coupling method. We performed the measurements of the electrochemical impedance for the conventional planar bilayer lipid membranes, *i.e.*, the monolayer holding method, to know the successful introduction of the anti-BSA antibody. The results obtained are shown in Fig. 2. The thickness of the hydrophilic region after the amine coupling step became larger, though the thickness of the alkyl chain part remained unchanged. This clearly indicates that the anti-BSA antibody as a receptor is able to be introduced by the amine coupling method.

Effect of the recording time on the integrated current

The effect of the recording time on the magnitude of an integrated current for gramicidin A without any receptor modification was investigated by recording the channel currents at –100 mV for 5 min. If channel events of gramicidin A, *i.e.*, the monomer/dimer formation, occurred at random and evenly, the magnitude of an integrated current would be independent of the recording time. Although the autopatch-clamp apparatus

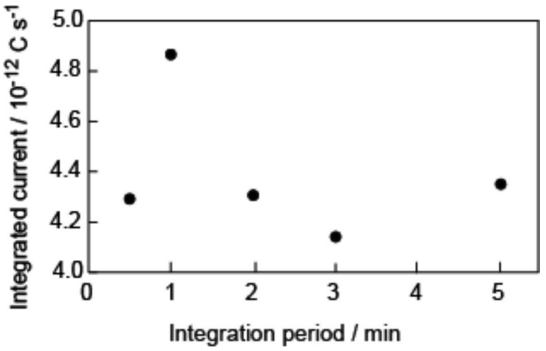

Fig. 3 Effect of the recording time on the integrated current of gramicidin A. Chamber solution, 0.15 M KCl containing 10 mM CH_3COOH/CH_3COOK (pH 4.0); applied potential (*cis* side), –100 mV.

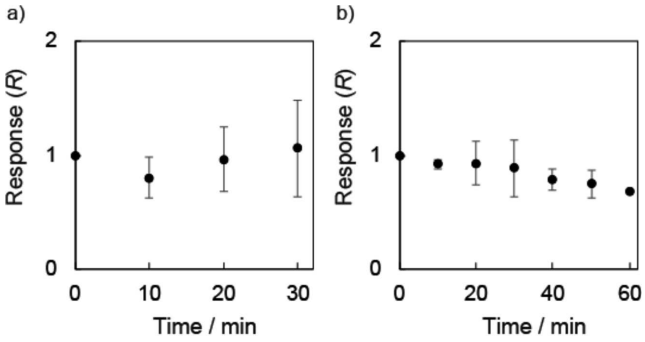

Fig. 4 Time course of the response with an anti-BSA-antibody modified membrane. The chamber solution and applied potential were the same as in Fig. 3. (a) A time course of the response during a flow of an acetate solution. (b) At $t = 0$, a flow of an acetate solution at 0.5 mL/min was stopped and channel currents were recorded in a static solution.

can form mechanically stable bilayers, a slight increase of the integrated current for a recording period of 1 min occurred due to the modulation of a lipid bilayer by adding gramicidin A and an insufficient number of channel opening events. As shown in Fig. 3, the integrated current became almost constant after channel currents were recorded for 2 min. This indicates that the channel events of gramicidin A occurred evenly during the period. Thus, recording currents to obtain a sensor response was performed for at least 2 min.

Stability of a bilayer lipid membrane

We investigated the stability of a lipid bilayer when receptor modifications were made on the surface of the lipid bilayer. Lipid bilayers formed by a miniaturized auto-patch system were mechanically stable, and the lifetime of the bilayers was at least above 40 h. The response of a bilayer lipid membrane after the modification of a receptor (anti-BSA antibody) in the flow system is shown in Fig. 4. Although the response (R) varied markedly from one membrane to another, indicating that perturbation of the bilayer lipid membrane occurred significantly by running an acetate solution (Fig. 4a), the response attained at a steady state when the flow was suspended (Fig. 4b). It appeared that the molecular alignment of the bilayer was slightly disturbed by running a solution, and it settled down gradually in a static solution. Consequently, the response of a bilayer lipid membrane sensor was necessary to be measured in a static solution.

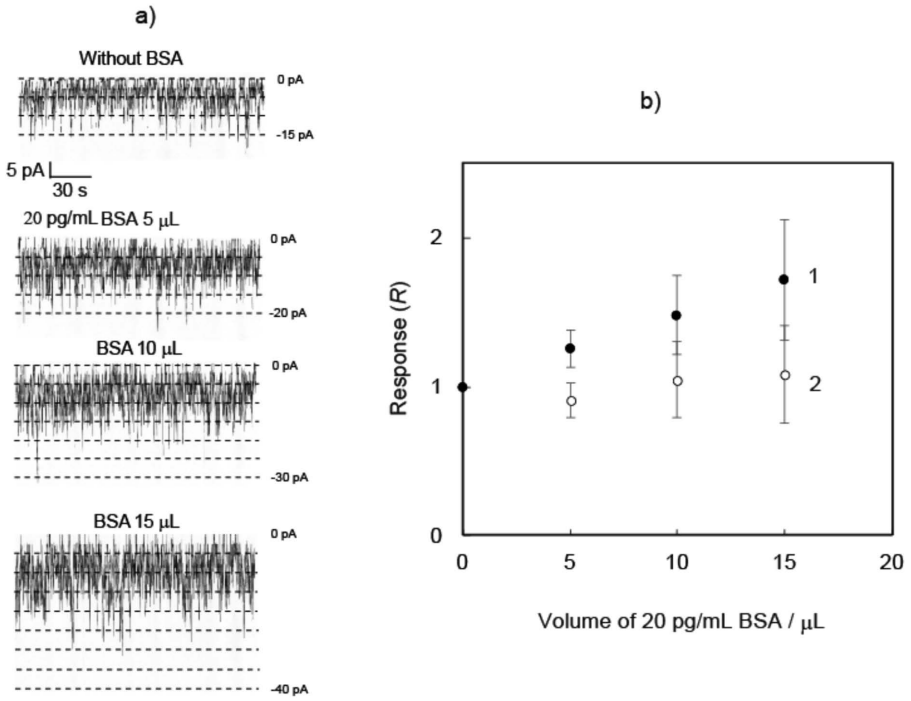

Fig. 5 Typical current traces and concentration dependence for BSA. Chamber solution, 0.15 M KCl containing 10 mM CH_3COOH/CH_3COOK (pH 4.0). Applied potential (*cis* side), –100 mV. (a) Typical current trace before and after the addition of BSA. (b) A plot of the response (R) against the concentration of BSA. (1) With anti-BSA-antibody (n = 3) and (2) without anti-BSA antibody (n = 3).

Concentration dependence for BSA

Since a horizontal bilayer lipid membrane is formed with an autopatch-clamp apparatus, only a chamber solution on the one side (*cis* side) of the bilayer was exchangeable, and a chamber was connected to the flow system. Hence, the volume of a *cis* side solution was not strictly defined. Hence, different volumes (5 - 15 μL) of a 20 pg/mL BSA solution were injected for obtaining a concentration dependence plot. Figure 5a shows the typical response of a bilayer lipid membrane at –100 mV to BSA in an acetate solution. The normalized response (R) increased with an increase in the added volume of a BSA solution at the level of pg/mL with a detection limit of (S/N = 3) (curve 1), as shown in Fig. 5b. Such changes in the response were not observed for the receptor-unmodified cases (curve 2).

The increase in the response (R) is explained by the presence of lipid rafts, which affect the channel activity of gramicidin A,[24] *i.e.*, the lipid mixture of DPhPC:DOPE:Chol (4:0.01:1, molar ratio) is not homogenous. The lipid rafts are likely to be modulated by the binding of BSA to anti-BSA modified on the DOPE head group, thereby the fraction of gramicidin A dimmer that stays in a DOPE($C_{18:1}$) raft increases and/or the distribution of potassium ions at the membrane surface is modulated. This may cause an increase of the gramicidin A channel conductance, leading to an increase in the integrated currents.

The effect of γ-globulin (~50 pg/mL) on the response of the sensor was investigated by adding the protein in the absence of BSA. The response to γ-globulin was much smaller as compared with that of BSA, indicating that the compound does not affect the response within the experimental error (Fig. S1, Supporting Information).

Conclusions

The results demonstrated above show that a channel-based biosensor based on the membrane bound receptor approach can be constructed with a miniaturized auto-patch system. The modernized bilayer formation technology improved the methodological difficulty of constructing a planar bilayer membrane. Although only an anti-BSA antibody was examined as a receptor, a variety of receptors can be employed for the development of a bilayer membrane sensor. Since the present study was focused on the optimization of an auto-patch system, selectivity issues were investigated only for γ-globulin as a typical protein existing in serum. It appears that appropriated tuning of the system provides a biosensor with selective and sensitive responses.

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Supporting Information

The effect of γ-globulin on the sensor response is shown in Fig. S1. This material is available free of charge on the Web at http://www.jsac.or.jp/analytsci/.

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