Stability of an Agar-Supported Bilayer Lipid Membrane and Its Application to a Chemical Sensor

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Lipid membranes made of lecithin and cholesterol were formed by self-assembly in a small aperture on an agar support. The membranes exhibited an average electric resistance of 135 GΩ and a capacitance of 0.43 μF/cm². Gramicidin, known to form a channel in uni-lamellar lipid bilayers, reduced the electric resistance to a MΩ level, thus showing the membranes to be of a uni-lamellar bilayer type. The membrane stability was investigated against perturbation with electric potentials and against mechanical agitation in the contacted aqueous solution. About 80% of the membrane preparations remained intact after applying electric potentials of between +1500 mV and −1500 mV. A similar percentage of the membranes stayed intact under 100 rpm magnet stirring in a 30 ml vessel. Membranes containing valinomycin responded to K^+ ions with changes in both the membrane conductance and the membrane potential.

Keywords Bilayer lipid membrane, lecithin, cholesterol, valinomycin, membrane conductance, membrane potential

Biological cell membranes, mainly consisting of proteins and lipids forming a uni-lamellar lipid bilayer, have many functions, such as molecular recognition, signal transduction and signal amplification.[1] It is therefore attractive to employ lipid membrane systems for chemical sensors. Recently, chemical sensing based on analyte-induced changes in the permeability through lipid membranes has been developed.[2–10] Changes in the permeability for electroactive marker ions, which were induced by chemical stimulations of Langmuir–Blodgett membranes[4–6] formed with receptor molecules and membranes self-assembled[7] on solid electrode surfaces, have been detected by measuring the redox currents for marker ions. Biological receptors have also been incorporated into bilayer lipid membranes (BLMs) formed by patch clamp[8] or folding[9,10] methods, and were used as sensing elements for determining the flux of electroinactive ions through these membranes. Minami *et al.* used ionophore-containing BLMs as sensing membranes, and reported on the potentiometric responses as a function of the controlled membrane-surface charges and the charge of analyte ions.[11]

Although artificial BLMs appear to be attractive as sensing membranes, the fragility of BLMs has limited their practical use. Attempts to overcome this have been made by incorporating stabilizing agents, such as surfactants and polymers[12,13], by miniaturizing the membranes[14,15] and by supporting the membranes with ion-conductive hydrated polymeric substrates.[16,17] Polymerized BLMs are of stable structure, but of low fluidity.[13] Thompson *et al.* reported that the lipid membranes formed in pores of microfiltration filters are resistant to physical shock and vibration.[15] Arya *et al.* developed polyacrylamide hydrogel-supported Langmuir-Blodgett membranes[16] and Hongyo *et al.* used agar-supported BLMs formed by self-assembly with successful BLM formation in 20% of all trials.[17] These supported BLMs had a mm level diameter and were relatively large compared with the BLMs formed by the patch-clamp and folding methods. The supports not only stabilize the BLMs, but also contain ions which may complex with the receptor of the BLM and, thus, be transported across the BLM. Hongyo *et al.* measured the membrane conductance in the ac mode because the ion content of the agar layer was too low to support an ion flux through the BLM in the dc mode. Supporting BLMs with an ion-conductive hydrated polymer is a simple method to stabilize BLMs, such as biological cell membranes which separates two aqueous phases. In this study, some modifications of agar-supported BLMs were attempted. Miniaturization and support of BLMs with an ion-conductive polymer layer, which was sufficiently large to measure the membrane conductance in the dc mode, were employed to form stable membranes with good fluidity.

BLMs made of lecithin and cholesterol were formed in a small aperture of 0.1 - 0.2 mm diameter in a 12.5-μm thick Teflon film supported by an agar layer containing an appropriate salt solution. The membrane stabilities were tested by applying an electrical potential and by stirring the sample solution. Valinomycin was incorporated as a receptor into these BLMs, and the response to K^+ was examined by measuring the membrane conductance and potential.

Experimental

Reagents and apparatus

Soybean lecithin and cholesterol from Wako Pure Chem. Ind. were used as received. A lipid solution was prepared by dissolving 100 mg of lecithin and 50 mg of cholesterol in 7.5 ml of hexane. Valinomycin and gramicidin were purchased from Sigma (St. Louis, MO, USA) and dissolved in hexane and ethanol, respectively. All other chemicals were obtained from Wako Pure Chem. Ind. Water was deionized and distilled.

Electrical currents between two Ag/AgCl electrodes were measured using a Nihon Kohden patch/hole cell clamp amplifier (CEZ-2300) and monitored with a Kenwood oscilloscope (CS-4035). Electrical potentials were applied using a Fuso potential sweep unit (Model HECS 980) with an amplifier. The membrane conductance and capacitance were calculated from the current changes upon applying a potential step and sweep, respectively (*vide infra*).

Potentiometric measurements were carried out with an Advantest (TR8411) vibrating-reed electrometer with a high input impedance ($>10^{15}$ Ω), which allowed measurements of the electrical potentials of membranes having resistances as high as 30 - 50 GΩ.[11]

The sample electrolyte solution was stirred with a 2 cm long Teflon-coated magnetic stirring bar driven from the outside of a Faraday cage with a magnetic stirrer.

Fabrication of agar supported bilayer lipid membrane electrodes

The agar-supported BLM electrode is schematically shown in Fig. 1. A circular aperture (0.1 - 0.2 mm diameter) in a 12.5-μm thick Teflon film was obtained by applying an electrical spark generated by an automobile ignition coil.[11,18] The shape and diameter of the aperture were confirmed under a microscope. The Teflon film was then washed with hexane and fixed with Teflon tape to the bottom of a plastic electrode body (5 cm in length and 1 cm in diameter). A hot aqueous agar (3%) solution containing an electrolyte (0.15 M KCl for evaluation of membrane stabilities, 0.15 M LiCl for the K^+ sensor) was poured into the electrode body, allowed 15 min to gell; then an Ag/AgCl electrode connected to an amplifier holder was inserted. Ten microliters of a lipid solution were spread on the small aperture. The electrode was immediately immersed into a 30 ml electrolyte solution (outer solution; containing the same electrolyte as the agar solution) and left standing for 15 min to allow for the self-assembly of a bilayer lipid membrane. The solution was gently stirred during this process. The yield of successful membrane fabrication by the present method was approximately 30% in the first 270 trials with a new Teflon film. A second Ag/AgCl electrode was also immersed in this outer solution.

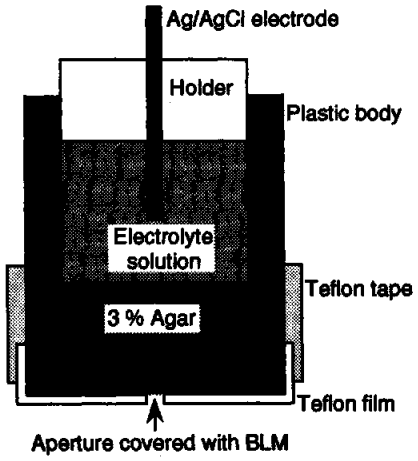

Fig. 1 Schematic illustration of an agar-supported BLM electrode.

In order to fabricate sensing membranes consisting of a BLM with a sensing element, valinomycin was dissolved in the lipid solution used to form the BLMs. As the electrolyte, 0.15 M LiCl was used instead of KCl.

Measurements of the membrane conductance and capacitance

A bilayer lipid membrane can be represented by a parallel combination of the membrane resistance and the membrane capacitance.[19] Electrical potentials of 0, +100, 0 and −100 mV were successively applied between the two Ag/AgCl electrodes for several minutes at each potential until a steady-state current was obtained. The membrane conductance was calculated based on the observed steady-state currents. The applied potential was swept linearly between 0 and 10 mV at a scan rate of 2 V/s, and the resulting currents were monitored with an oscilloscope. The membrane capacitance was calculated from the current changes observed upon reversing the sweep direction.

Results and Discussion

Fabrication of BLMs

The mean electrical resistance and the capacitance of the membranes were found to be 135 GΩ (n=85, SD=63) and 0.43 μF/cm² (n=28, SD=0.22), respectively. These values are comparable to those for uni-lamellar bilayer lipid membranes formed by the patch-clamp[8,19] or folding method.[9,10] A 0.1 ml portion of a 1 μg/ml gramicidin solution was added to the 30 ml outer solution.

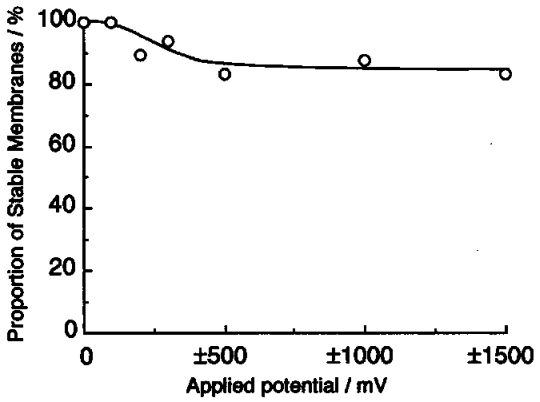

Fig. 2 Stability of an agar-supported BLM (lecithin/cholesterol weight ratio, 2 : 1) against the applied electric potential. Potentials of 0, x, 0 and $-x$ mV were successively applied for 5 min at each potential, varying x between 0 and 1500 mV. The stability was defined as the percentage of the number of membranes remaining intact after potential application.

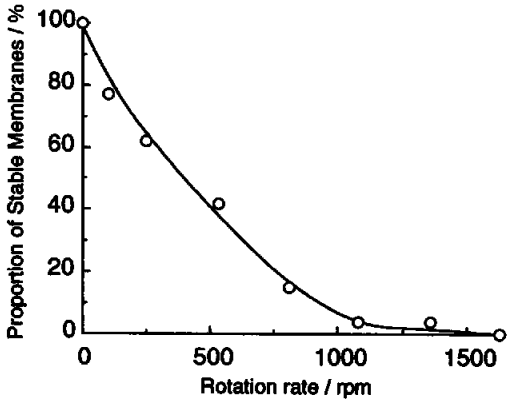

Fig. 3 Stability of an agar-supported BLM against stirring of the sample solution. The outer electrolyte solution was stirred for 5 min at each rotation rate. The definition of the stability was the percentage of the number of membranes remaining intact after stirring the solution.

Gramicidin, known as a channel former with its dimer form spanning across a bilayer lipid membrane, reduced the membrane resistance to the MΩ level. On the basis of these results, it is concluded that agar-supported lipid membranes are actually uni-lamellar bilayer lipid membranes.

To evaluate the stability of agar-supported bilayer lipid membranes, electric potentials were applied to the membrane and the outer solution was stirred. Here, the membrane stability was defined as the percentage of membranes preserved intact after potential application or perturbation by stirring.

Figure 2 shows the stability of the membranes against the application of electrical potentials. For each experiment, the potential was set successively to a positive and a negative value for 5 min. The membrane conductance was then measured again. In 15 out of 18 runs the membrane exhibited no changes in conductance, even after potential applications of +1500 mV or −1500 mV; such large potentials have been conventionally used to break any BLMs formed by the patch-clamp method. It is thus clear that agar-supported membranes are more stable towards applied electrical potentials than BLMs formed between two aqueous solutions.

The effect of the stirring rate of the outer solution on the stability of the membrane, examined in 26 independent runs, is shown in Fig. 3. The proportion of stable membranes decreased along with an increasing stirring rate. Since the BLM is an assembly of amphiphilic lipid molecules held together by noncovalent interactions, lipid molecules might be gradually pulled out from the BLM by vigorous stirring of the solution; finally, the number of lipids could become too small to maintain the membrane structure in the aperture. However, 77% of the membranes remained stable at 100 rpm stirring. On this basis, the solution was stirred at 100 rpm in subsequent experiments.

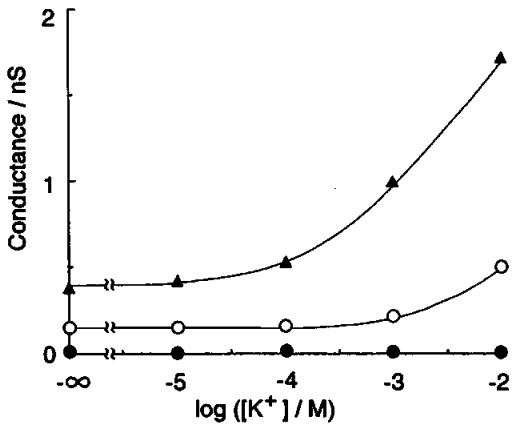

Fig. 4 Concentration dependence of the membrane conductance for an agar-supported BLM. The compositions of the BLMs were lecithin, cholesterol and valinomycin with a weight ratio of 2 : 1 : 0 (●), 2 : 1 : 0.02 (○) and 2 : 1 : 0.1 (▲).

Fabrication of K^+ sensor

The present agar-supported BLM was used in a K^+ ion sensor based on valinomycin by measuring changes in the membrane conductance and membrane potential. The membrane was of a lecithin/cholesterol/valinomycin weight ratio of 2 : 1 : 0, 2 : 1 : 0.02 or 2 : 1 : 0.1, and the sample solution contained 0.15 M LiCl.

The membrane conductance of the agar-supported BLMs responded to K^+, reaching a steady-state value after stirring for 5 min. Indicated in Fig. 4 are the average conductance values measured at positive and negative applied potentials of equal amplitude. Averaging was made because positive and negative potentials yielded almost the same conductances. The differences

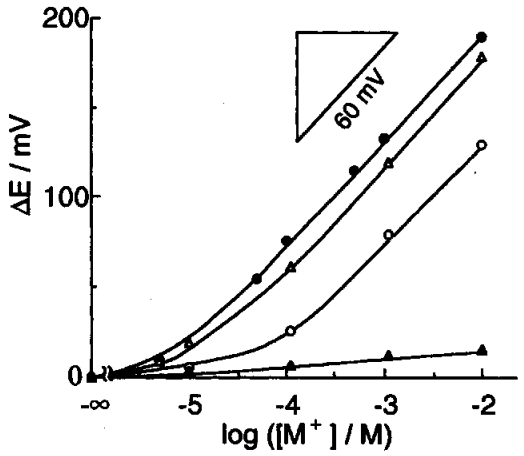

Fig. 5 Potentiometric response of an agar supported lecithin/cholesterol/valinomycin (2:1:0.1, w/w) BLM to Rb^+ (●), K^+ (△), Cs^+ (○) and Na^+ (▲) in 0.15 M LiCl. ΔE is the difference of potentials in the presence and absence of alkali metal ions.

in the membrane conductance between the three BLMs in the absence of K^+ may be caused by the formation of a weak complex of valinomycin and Li^+. The membrane conductance increased along with the K^+ concentration, and also depended upon the concentration of valinomycin. Hongyo *et al.* used an ac excitation for the measurement of the membrane conductances in order to prevent the depletion of ions in the small (*ca.* 1 μl) agar layer.[17] In our case, the amount of ions in the agar layer was sufficiently large to supply ions for dc excitation, resulting in a steady-state response of the sensor. The ion flux through the membrane was not only due to K^+ carried by valinomycin, but also other ions, such as Cl^- and Li^+. This can be seen from the fact that the observed currents at both positive and negative potentials increased along with increasing concentration of K^+, added only on one side of the membrane, even though the direction of the ion flux was reversed when changing from positive to negative potentials.

After the addition of alkali metal ions and stirring for 2 min, the membrane potential of the agar-supported BLMs reached a steady state within 2 min. The membrane potential response to Rb^+, K^+ and Cs^+ was Nernstian (Fig. 5). The slope of the log C_{K^+} *vs.* ΔE for K^+ was 60.0 mV/decade with a correlation coefficient of 1.00 in the concentration range from 10^{-4} to 10^{-2} M and a relative standard deviation of the measurement at 10^{-3} M of K^+ of 2.0% (n=3). The selectivity coefficients of this system, determined by the matched potential method, were 1.75 and 0.20 for Rb^+ and Cs^+, respectively. These results are comparable to those of conventional liquid membrane ISEs[20] and those of a bilayer lipid membrane formed by the folding method.[11]

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References

1. B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts and J. D. Watson, "*Molecular Biology of The Cell*", Chap. 6, Garland Publishing, New York, 1983.
2. K. Odashima, M. Sugawara and Y. Umezawa, *Tr. Anal. Chem.*, **10**, 207 (1991).
3. D. P. Nikolelis and U. J. Krull, *Electroanalysis*, **5**, 539 (1993).
4. M. Sugawara, K. Kojima, H. Sazawa and Y. Umezawa, *Anal. Chem.*, **59**, 2842 (1987).
5. S. Nagase, M. Kotato, R. Naganawa, R. Komatsu, K. Odashima and Y. Umezawa, *Anal. Chem.*, **62**, 1252 (1990).
6. M. Sugawara, H. Sazawa and Y. Umezawa, *Langmuir*, **8**, 609 (1992).
7. N. Nakashima, T. Taguchi, Y. Takada, K. Fujio, M. Kunitake and O. Manabe, *J. Chem. Soc., Chem. Commun.*, **1991**, 232.
8. M. Uto, E. K. Michaelis, I. F. Hu, Y. Umezawa and T. Kuwana, *Anal. Sci.*, **6**, 221 (1990).
9. H. Minami, M. Sugawara, K. Odashima, Y. Umezawa, M. Uto, E. K. Michaelis and T. Kuwana, *Anal. Chem.*, **63**, 2787 (1991).
10. N. Sugao, M. Sugawara, H. Minami, M. Uto and Y. Umezawa, *Anal. Chem.*, **65**, 363 (1993).
11. H. Minami, N. Sato, M. Sugawara and Y. Umezawa, *Anal. Sci.*, **7**, 853 (1991).
12. P. K. Shieh and L. Packer, *Biochem. Biophys. Res. Commun.*, **71**, 603 (1976).
13. H. H. Hub, B. Hupfer, H. Koch and H. Ringsdorf, *Angew. Chem. Int. Ed.*, **19**, 938 (1980).
14. J. M. Mountz and H. T. Tien, *Photochem. Photobiol.*, **28**, 395 (1978).
15. M. Thompson, R. B. Lennox and R. A. McClelland, *Anal. Chem.*, **54**, 76 (1982).
16. A. Arya, U. J. Krull, M. Thompson and H. E. Wong, *Anal. Chim. Acta*, **173**, 331 (1985).
17. K. Hongyo, J. Joseph, R. J. Huber and J. Janata, *Langmuir*, **3**, 827 (1987).
18. R. Hartshorn, M. Tamkun and M. Montal, in "*Ion Channel Reconstitution*", ed. C. Miller, Chap. 8, Plenum Press, New York, 1986.
19. A. Marty and E. Neher, in "*Single-Channel Recording*", ed. B. Sakmann and E. Neher, Chap. 7, Plenum Press, New York, 1983.
20. K. Umezawa and Y. Umezawa, in "*CRC Handbook of Ion-Selective Electrodes: Selectivity Coefficients*", ed. Y. Umezawa, CRC Press, Boca Raton, 1990.

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