SIGNIFICANT IMPROVEMENT IN SENSITIVITY OF LEAKAGE CURRENT MICROSENSOR BY USING DENATURANT AND ELECTROLYTE-ENTRAPPING DPPC LIPOSOMES

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ABSTRACT
We have developed a highly-sensitive leakage current microsensor by using DPPC liposome entrapping K₄[Fe(CN)₆] solution to detect existence of biomolecules especially proteins and their dynamic conditions. In this work, the addition of guanidinium hydrochloride (GuHCl) as protein denaturant successfully leads to obtain a prominent improvement although target protein, which is carbonic anhydrase from bovine (CAB) and lysozyme, weakly interacts with liposome under the normal condition. Moreover, we can use it to evaluate the conformation state of CAB and lysozyme protein such as ‘Native’, ‘Molten-Globule’ and ‘Unfold’ states.

KEYWORDS: Entrapping liposome, Guanidinium hydrochloride, Carbonic anhydrase from bovine, Lysozyme

INTRODUCTION
We have reported in our previous work [1] that the liposome entrapping electrolyte such as ferrocyanide (Fe(CN)₆³⁻) ions can release the ions by interaction with biomolecules (such as proteins) through their structural change and the membrane perturbation [2,3]. Leakage current was measured on a basis of DC amperometry. The released ions can contribute to the current generation, which relates directly to the interaction between liposome membranes and external proteins. However, the possibility of improvement of the leakage current sensitivity against the target protein detected in our microsensor has remained unclear.

In this paper, compared to our previous work [1], we have newly developed a highly-sensitive leakage current microsensor by using the entrapping DPPC liposome and a protein denaturant. The addition of denaturant successfully resulted in the prominent improvement in sensitivity up to 129-fold of magnitude, although the target protein weakly interacts with liposome under the normal condition.

THEORY
The lipid membrane of the DPPC liposome is perturbed by the interaction with the external protein, thereafter iron ion Fe²⁺ is released from K₄[Fe(CN)₆] solution inside of the DPPC liposome through the perturbed lipid membrane. The Fe²⁺ ion is oxidized to Fe³⁺ and generated electron goes to the anode. The series phenomena are illustrated in Fig. 1. The leaked Fe²⁺ ion shows an electrochemical reaction in neighbor of the anode in (1);

\[
\text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + e^-, \quad (1)
\]

and the Fe³⁺ ion goes to the cathode and is reduced to Fe²⁺ receiving an electron. The generated electron gives the leakage current in the microsensor. The leakage current is dependent on the quantity of Fe²⁺ ions leaked from K₄[Fe(CN)₆] solution inside of the DPPC liposome, so this means that one can obtain a quantitative intensity of interaction between the DPPC liposome and protein, such as an external protein concentration and activity of the interaction.

EXPERIMENTAL
Device fabrication
The fabrication process of leakage current sensor is shown in Fig. 2. A 280 μm thickness Si wafer with 1 μm thickness SiO₂ layer is used as an initial substrate. At first, a micro-well that will hold a droplet of entrapping DPPC liposome steadily is formed by BHF etching of SiO₂ film and TMAH anisotropic wet etching of the Si bulk. After that, the surface of the well are thermally oxidized to eliminate external leakage current between the sensor electrodes and to have a hydrophilic surface for keeping intact molecular structure of the liposome. In the last process, Pt/Ti film
electrodes are formed by rf-sputtering and lift-off method. The chip size, the depth of the well and the widths of electrodes are about 14 mm, 100 μm and 500 to 2000 μm, respectively.

Results and Discussion

A consideration of the sensor system is done on the biochemical solution used in the leakage current microsensor such as droplet volume of liposome and protein, and entrapping liposome concentration. By increasing droplet volume of biochemical solution, the leakage current increases non-linearly and have saturation point around 2 μL as shown in Fig. 5. After that, we increased the entrapping DPPC liposome concentration. In Fig. 6 the leakage current increases non-linearly and have saturation point over 2 mM as a similar manner in Fig. 5. It is considered that the leakage current is sufficiently correlated with both the droplet volume and entrapping liposome concentration but has saturation points which limit the current. From these results, at this stage, we selected the droplet volume more than 1 μL and the entrapping DPPC liposome more than 2 mM to obtain the clear interaction peak of leakage current from nA to μA order in the sensor system.

In this experiment we used 10 mM entrapping DPPC liposome concentration and 1 μL of droplet volume. We monitored the time-course of the leakage current with 0.1 V bias between the electrodes as shown in Fig. 7. From the literature [3], the peak leakage current corresponds to the generated charge of Fe^{2+} ions released by the interaction between the liposomes and CAB. The peak value of leakage current was plotted as a function of the GuHCl concentration (Fig. 8), showing the addition of GuHCl (up to 2 M) made the prominent improvement of the leakage current possible. The improvement of sensitivity by 129-fold was observed at 1.5 M of GuHCl (Fig. 9). We considered that the sensitivity improvement resulted from the interaction between liposome and GuHCl-induced CAB at Molten-Globule (MG) state, consistent with the finding that CAB at MG state (at around 1.5 M of GuHCl) can strongly
interact with liposome membranes. The above detection results are consistent with the previous report with respect to the conformation of CAB using a conventional chromatography setup of laboratory instruments [3], as seen in Fig. 10. From these results, we applied the sensor also for detecting lysozyme protein as shown in Fig. 8. It is found that the addition of GuHCl concentration (up to 2 M) has improved the sensitivity of the sensor and the lysozyme protein becomes easier to detect at Molten-Globule (MG) state (at around 1.5 M of GuHCl) than the normal condition (without GuHCl). The results are consistent with the above CAB results.

CONCLUSION

We have successfully fabricated and measured a specific biomolecule microsensor for an evaluation of a leakage current in liposome solution. A clear leakage current in 0.1 μA order was able to be detected from the denatured protein-liposome interaction. The sensitivity of the leakage current microsensor was significantly improved due to the enhancement of protein-liposome interaction. The denaturant-based improvement of the detection of target protein is a promising strategy for the design and development of the microsensor in the integrated microsystems and also enables to detect other proteins that do not have large interaction with the liposome.

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