IMMOBILIZED-LIPOSOME SENSOR SYSTEMFOR DETECTION OF DAMAGED PROTEINS

R. Kuboi*[1], H. Umakoshi*, T.Shimanouchi*, H.Ishii*,V.T.Huong* and S. Morita***

* *Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama-cho, Toyonaka, Osaka, 560-8531, Japan*

*** *Department of Material Science, Wakayama National College of Technology, Noshima 77, Noda-cho, Gobo, Wakayama, 644-0023, Japan*

1: Corresponding author

ABSTRACT

An electrolyte-entrapping liposome was immobilized on an Au electrode to design and develop of an immobilized liposome sensor (ILS) system for the detection of the damaged proteins. Three proteins (bovine carbonic anhydrase, lysozyme and chitosanase) were used as model proteins. Damaged proteins could be effectively detected by a chronoamperometric method due to the protein-liposome interactions. The ILS system can be a useful tool for an on-line monitoring to not only conformational changes of proteins but also a bioprocess.

KEYWORDS

Liposome, Immobilized-liposome sensor, Protein-lipid interaction, Stress condition

INTRODUCTION

Damaged proteins and peptides such as the conformational abnormality of protein, has recently be recognized as a certain toxic to the cell tissues (Hardy and Higgins, 1992, Shimanouchi et al., 2007). The causative mechanism is considered to be the interaction of damaged proteins with the normal or damaged biomembranes. The evaluation of the above interaction has bee desired but there is little report in this field. For this purpose, liposome is the appropriate model system because the liposome is composed of a closed bilayer phospholipid membrane that has previously been used as a *nano-artificial cell membrane*, and because the preparation of the damaged liposomes can be easily peformed by using the chemical or physicochemical tequniques.

Since an immobilization technique was developed by Lundahl and Yang (1991), liposomes have been used as sensor elements in various monitoring methods, such as surface plasmon resonance (SPR) (Yamaguchi et al., 2003), immobilized liposome chromatography (ILC) (Yoshimoto et al., 1998, 2006, Liu et al., 2001), quartz crystal microbalance analysis (QCM) and dielectric dispersion analysis (DDA)(Morita et al., 2003). These tequniques gives us the valuable information on the protein-liposome interaction. It is a common point of these methods that the liposomes are immobilized as sensor elements on a detector surface or a gel support and that the liposome-protein interaction can be directly evaluated. In general, the detection principal is based on the adsorption of protein on the liposomal surface or the retardation of elution behavior, resulting from the interaction between liposomes and proteins.

A liposome entrapping calcein has recently been developed to improve the sensitivity of ILC (Liu et al., 2001). It is suggested that calcein release is a useful index for the detection of the protein-liposome interaction because the release of the calcein is affected by membrane characteristics such as membrane fluidity and permeability (Kuboi et al., 2004). An electrolyte-entrapped immobilized-liposome electrode has recently been developed and applied to the analysis of the protein-lipid membrane interaction using the amperometry (Jung et al., 2003, 2007). These methods are based on the release of detectable materials entrapped in an immobilized liposome.

The electrochemical technique is the useful measurement method prior to the sensitivity of signal and its quick response.

The purpose of this study is to clarify the possibility if the immobilized liposome sensor system based on the electrochemical method can be a useful tool for the study on the damaged protein-liposome membrane interaction. We first investigated the eelectrochemical response in our sensor system in the presence of proteins at various guanidium chloride (GuHCl) concnetrations. We also investigated the surface properties (local hydrophobicities) of proteins in connection with the above results.

MATERIALS AND METHODS

1-palmitoyl-2-oleoyl-sn-grycero-3-phosphocholine (POPC)/ egg yolk phosphatydyl-ethanolamine (EPE) liposomes were prepared as previously described (Yoshimot et al., 1998). In brief, POPC(9.8 mg/ml)/EPE(0.2 mg)/chitosan(1 mg) dissolved in chloroform were dried in a 100 ml round-bottom flask by rotary evaporation under reduced pressure. The lipid film was dissolved in diethylether twice, and the solvent was evaporated again. The obtained lipid film was kept under high vacuum for at least 3 hr and then hydrated with the 50 mM potassium hexacyano-ferrate (II) solution in 50 mM potassium phosphate buffer (pH 7.5) at room temperature to form multilamellar vesicles. After the freeze-thawing treatment, the liposome size was adjusted by the extrusion of the solution through polycarbonate membranes (diameter 100 nm).

To fabricate the immobilized liposome electrode, a self-assembled monolayer (SAM) using 1,6-mercaptohexadecanoic acid was formed on Au electrode (2 mm×2 mm). To activate the SAM membrane, the above electrode was immersed in solution consisted of dioxane; distillied water (90:10) solution contained 17 mM N-hydrosysuccinimide, 17 mM WSC for 3~4 hr. The liposome was then formed on the SAM layer by the amino conjugate method. After 1 hr, the liposome formed Au electrode was rinsed with phosphate buffer (pH7.5). A potentiostat/Galvanostat (EG&G model 273) connected to a personal computer (EG&G Software Power Suit #270/250) were used for the electrochemical measurements. A conventional three-electrode cell, consisting of the Au electrode modified with liposome as a working electrode, a platinum wire as a counter electrode modified with liposomes as a working electrode used for the electrochemical measurement.

RESULTS AND DISCUSSION

Immobilization of liposome on the electrode for sensor element

The electrolyte-entrapping liposomes were immobilized on the electrode by the method for the covalent coupling using self assembled monolayer (SAMs) of 1,6-mercaptohexadecanoic acid as shown in Fig. 1. From an atomic force microscopy (AFM) observations, the physical morphology of Au electrode is considered to have a flat surface and the average height roughness is approximately 2 nm. For the electrodes immobilized liposomes, relatively uniform liposome layer was found to be formed although the electrode surface was not completely covered by liposomes(data not shown). We checked the immobilization of liposomes onto the electrode with a cyclic voltammogram. The immobilization of liposomes drastically decreased the peak current derived from the redox process (data not

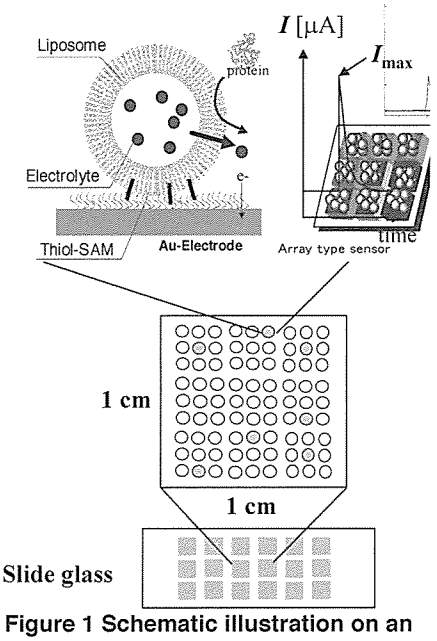

Figure 1 Schematic illustration on an immobilized-liposome sensor system.

shown). This suggests the existence of a layer inhibiting the redox process on the electrode surface. Furthermore, the addition of the surfactant Triton X-100 (5 mM) induces the significant change in current due to the disruption the membrane structures. Based on the above results, it is concluded that the liposomes could be immobilized onto the electrode with keeping their structures.

Electrochemical response by protein addition

The output current of electrode upon various concentrations of guanidine hydrochloride (GuHCl) is shown in Fig. 2(a). The instantaneous increase in current and the subsequent gradually decrease according to Cottrell's equation ($i(t) = nFDc/(\pi Dt)^{0.5}$; i: cathodic current density [A/m^2], n: number of electrons [-], F: Faraday's constant [C/mol], D: diffusion constant for electrolyte [m^2/sec], c: electrolyte concentration [mol/m^3], t: time [s]) were observed. Since n = 1 was yielded from the electrode reaction: $[Fe(CN)_6]^{4-} \rightarrow [Fe(CN)_6]^{3-} + e$, $D = 7.33 \times 10^{-10}$ m^2/sec (0.5 M of GuHCl) and 5.22×10^{-10} m^2/sec (2.0 M of GuHCl) were obtained. These data are consistent with previous literature: 6.18×10^{-10} m^2/sec (1 mM $[Fe(CN)_6]^{4-}$, 0.5 M KCl, 25°C)(Hyk et al., 2002). The diffusion constant was larger than that of protein estimated by Einstein-Stokes equation, indicating that the electric current was not the result of the migration of protein molecules.

Based on these results, the diffusion current (I_{max}) was utilized as the indicator of this sensor system in the following section.

Sensing of damaged protein under stresses

The response behavior of POPC/chitosan electrode on the denatured CAB in the presence of GuHCl was studied. The output current of electrode upon various concentration of GuHCl is shown in Fig.2(b). Conformational change of CAB was controlled by the addition of 0-2M GuHCl in eluent and it should be noted that CAB was pre-equilibrated with GuHCl solution for 2 h before applying to electrode. The maximum current at 1M GuHCl was observed. The peak value of current normalized by CAB concentration and its LH_{pr} value were plotted against the GuHCl concentration (Fig. 2(b)). Only a single peak for both I_{max}/C_{pr} and LH_{pr} was observed at around 1 M GuHCl and the change in the I_{max}/C_{pr} value is compatible with that in the LH_{pr} value, strongly suggesting that local hydrophobic sites of CAB specifically interact with liposomal membranes. It has been reported that proteins only at the MG state can interact with lipid membranes. The membrane permeability of liposome has immediately been increased because of the induction of the perturbation or fluctuation of membrane structures when the protein of MG state interacts with membrane surface. Then, it is considered that POPC/chitosan electrode can electrochemically recognize the conformation of CAB with high local hydrophobicity.

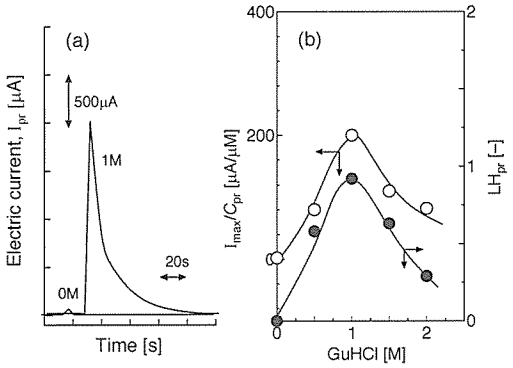

Figure 2 (a) Response of POPC/Chitosan electrode on the denatured CAB in the presence of GuHCl. (b) GuHCl-concentration dependencies of current and LH_{pr} of CAB. C_{pr} = 10 μM, Detection condition is +0.290 V (vs. Ag/AgCl)

Evaluation of the liposome electrode-proteins interaction

The detected signal for the three proteins examined in this study (CAB, lysozyme and chitosanase) at various states (i.e. native, reduced and partially denatured states) was compared in relation to their surface properties. Figure 3 shows the dependence of the surface properties (LH_{pr}) on the maximuma electric current, determined by using the immobilized-liposome sensor system. Although three proteins were used, a single linear relationship between

LH_{pr} value and maximum electric current normalized by protein concentration (I_{max}/C_{pr}) was observed in the range of LH = 0 to 1.6 regardless of proteins and the species of stresses (heat or chemical reagents). The similar relationships were also observed in the other lipid composition such as POPC and POPC/cholesterol liposomes. In the higher LH_{pr} value range, the experimental data was deviated from the desired line. This might result from the contribution of hydrogen bonding stability of protein molecule. Conformational stability of protein molecule is mainly dominated by both hydrophobic interaction and hydrogen bonding stability (Fernandez and Berry, 2003). This result showed the possibility of the quantification of hydrogen boning stability of protein.

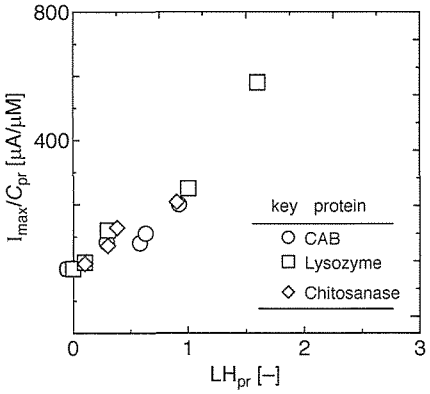

Figure 3 The normalized current as a function of LH_{pr} of two proteins. C_{pr} = 10 μM, Detection condition is +0.290 V (vs. Ag/AgCl)

Then a new detection system for the on-line monitoring of the stress-responsive bioprocess under the stress condition has thus been developed. As a result, the sensor chip using an electrolyte-entrapping liposome may be a useful tool for the detection of structural change of protein under the stress conditions as well as the conventional sensor systems such as ILC which is also based on the detection of the liposome-protein interaction (Yoshimoto et al., 2006).

CONCLUSIONS

We developed the immobilized-liposome sensor system for the detection of the stress-induced structural change of proteins using electrolyte-entrapping liposome electrode. The immobilized-liposome sensor gave us the quantitative data to support that the protein-liposome interaction was mainly predominated by hydrophobic interaction. We finally discussed the importance of the contribution of hydrogen bonding stability against the protein-liposome interaction.

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