DESIGN AND DEVEOLPMENT OF NANO-ARTIFICIAL-CELL MEMBRANE BASED NOVEL BIOSENSOR –APPLICATION FOR MONITORING OF AQUEOUS STRESSES-

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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 aqueous stresses. Two proteins bovine carbonic anhydrase and lysozyme were used as model aqueous stressors. 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

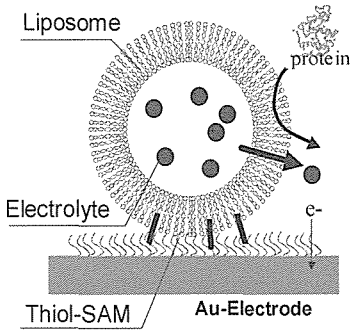
Liposome, Immobilized-liposome sensor, Protein-lipid interaction, Stress condition

INTRODUCTION

Studies of the function of cell membranes have attracted many researchers because environmental signals (stimuli or stress) are normally recognized on the membrane surface and responses of the cell are induced there. A method of evaluating valuable information on the membrane surface, such as signal induction, virus infection, and morphological changes of the cell membrane (endocytosis/exocytosi), has recently been presented using new techniques (Huttner and Schmidt, 2002). Currently, the idea that membrane–protein interaction triggers membrane-related phenomena has become mainstream in research on the cell membranes (Schmidt et al., 1999). Many researchers have previously addressed the structure and functions of (membrane) proteins related to these phenomena. The significance of the role of the cell membrane, including the phospholipid bilayer, has gradually been recognized (Jung et al., 2003). In such a sense, damaged proteins is thought to be the aqueous stressors as well as the environmental stresses (i.e. pHs, chemical reagents and heat). An intermittent heat stress can enhances the productivity of the target materials in the bioprocesses. It is therefore important and necessary to design and develop an on-line detection system for membrane-protein interactions to quantify the function of the cell membrane and to apply to the bioprocess.

Liposome is composed of a closed bilayer phospholipid membrane that has previously been used as a *nano-artificial cell membrane*. 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, Liu et al., 2001), quartz crystal microbalance analysis (QCM) and dielectric dispersion analysis (DDA)(Morita et al., 2003). 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 amperometry (Jung et al., 2003). These methods are based on the release of detectable materials entrapped in an immobilized liposome. Although there are many reports on this issue, a common mechanism of release has not been presented because of the lack of basic knowledge about the relationship between the membrane properties and the release behavior.



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

The purpose of this study is to obtain fundamental data on the release of detectable materials from immobilized liposomes to design a liposome-based stress sensor effectively. We first investigated electric current from liposomes in the presence of proteins at various guanidium chloride (GuHCl) concnetrations. We also investigated the surface properties (local hydrophobicities) of proteins to compare the above results. The effect of lipid composition and protein type on the calcein release was also studied. Based on these results, a possibility on tan immobilized-liposome sensor system was finally discussed.

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 Figure 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 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, the liposomes are concluded to be immobilized onto the electrode with keeping their structures.

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 Figure 2(a). 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 (Figure 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.

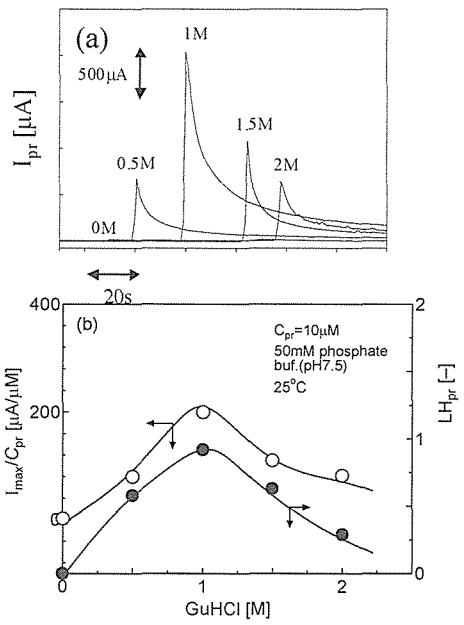


Figure 2 (a) Response of POPC/Chitosan electrode on the denatured CAB in the presence of GuHCl. (b) GuHCl-concentration dependencies of current and LH_{pr} of CAB. C_{pr} = 10 μM, Detection condition is +0.290 V (vs. Ag/AgCl)

Evaluation of he liposome electrode-proteins interaction

The detected signal for the two proteins examined in this study (CAB and lysozyme) 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 two 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. The similar relationships were also observed in the other lipid composition such as POPC and POPC/cholesterol liposomes.

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.

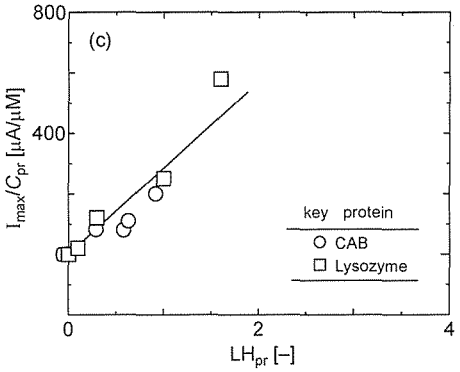


Figure 3 The normalized current as a function of LH_{pr} of two proteins. C_{pr} = 10 μM, Detection condition is +0.290 V (vs. Ag/AgCl)

CONCLUSIONS

We developed the immobilized-liposome sensor element with high stability for the detection of the stress-induced structural change of proteins using electrolyte-entrapping liposome electrode.

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