Multiphase material containing poly(L-glutamate) for biomembrane mimetic sensors*

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Since membrane-bound macromolecules provide the most general and powerful mechanisms for biological ion sensing and ion channelling, a new type of polymer-modified electrode was designed: a multiphase material consisting of poly(styrene-*co*-acrylonitrile) and poly(L-glutamate) was synthesized as the block copolymer. The material has enabled polypeptides to be immobilized onto electrodes just by dipping. Electrochemical responses towards Ca^{2+} were obtained based on the permeability change of the redox-active species as the indicator. The modified electrode has remarkable stability due to the vinyl polymer chains which serve as building blocks of the membrane.

(Keywords: poly(L-glutamic acid); calcium ion sensor; ion channel mechanism; *FT*i.r.)

INTRODUCTION

Signal transduction at cell surface receptors has been known to occur by one of three basic mechanisms, which are seen in ion channel systems, second messenger systems and receptors with integral enzyme activity[1]. These unique mechanisms in biological sensing would be a good example when designing a new principle in chemical sensor technology.

In this context, the concept of 'ion-channel sensors' developed by Sugawara *et al.*[2] is of much interest since the sophisticated mechanism occurring in cell membranes is successfully mimicked: a lipid-made Langmuir–Blodgett membrane coated on a solid electrode functions as an 'ion channel' which can be 'opened' specifically by the analyte ion, resulting in the permeability change of the electrochemically detectable ions as the indicator. The electrode has responded to 10^{-3} M of Ca^{2+}. Nakashima *et al.* have reported an 'ion-gate' lipid monolayer membrane which showed electrochemical responses to pH based on a similar mechanism[3].

In cell membranes, on the other hand, lipid molecules mainly form a permeation barrier and thereby establish cell compartments, whereas membrane proteins are responsible for most of the dynamic processes including ion sensing and ion channelling. In biological systems, membrane-bound macromolecules thus provide the most general and powerful mechanisms for chemical and physical signalling processes. Macromolecular components rather than lipids should, therefore, be utilized much more extensively in developing chemical sensors. However, though polymeric materials are widely used in sensor technology, few of them play a key role in sensing[4–6].

Our current interest has concentrated on taking advantage of synthetic macromolecules as a receptive component of electrochemical sensors[7]. We describe in this paper a biomembrane-mimetic sensor for the Ca^{2+} ion. A stable polymer membrane bearing synthetic polypeptide as a chemoreceptive component was prepared on a platinum wire. Electrochemical responses towards 10^{-6} M Ca^{2+} were obtained based on the permeability change of the redox-active couple ions. The mechanism for the response is proposed on the basis of *FT*i.r. measurements and electrochemical experiments.

EXPERIMENTAL

Materials

Poly(styrene-*co*-acrylonitrile) (3 g, $M_n = 6000$, styrene: acrylonitrile = 5:1) having a terminal carboxyl group, which was supplied by Toagosei Chemical Industry Co. Ltd, was dissolved in thionyl chloride (8 g) and the mixture was heated at 80°C for 7 h. Then the mixture was dried *in vacuo*, and the resulting solid was dissolved in dry benzene (5 ml). The benzene solution was poured slowly into dry benzene (100 ml) containing ethylenediamine (3.5 g) with stirring. The mixture was stirred overnight, and concentrated to ~10 ml. The solution was poured dropwise into methanol (300 ml) with stirring at room temperature. The precipitate was dissolved in benzene and reprecipitated into methanol; this procedure was repeated three times in total. The precipitate was dried to give white powder (1.6 g). The i.r. spectrum of the polymer showed an absorption at 1660 cm^{-1} due to the amide bond, but the absorption at 1705 cm^{-1} due to the terminal carboxyl group disappeared.

The primary amino group-initiated polymerization of γ-methyl L-glutamate *N*-carboxyanhydride (NCA, donated by Ajinomoto Co. Ltd) was carried out to

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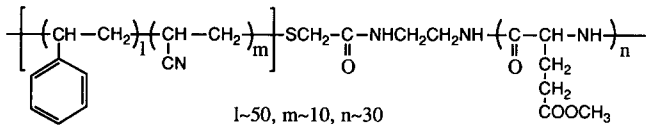

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Figure 1 Structure of the polymer prepared

yield a block copolymer, poly(styrene-*co*-acrylonitrile)-*b*-poly(γ-methyl L-glutamate) (**1**, see *Figure 1*). A tetrahydrofuran solution (5 ml) of NCA (0.3 g) was introduced in 1,2-dichloroethane (25 ml) of the polymer (0.3 g) and stirred at room temperature overnight. The i.r. spectrum of the reaction mixture showed the disappearance of the peaks due to NCA; the spectrum showed some typical absorption bands assigned to poly(γ-methyl glutamate) as well as polystyrene and polyacrylonitrile. The degree of polymerization of the polypeptide segment was 30 as estimated by ^{1}H n.m.r. in trifluoroacetic acid as solvent. No further purification was carried out for use in the electrode modification; the reaction mixture was diluted with 1,2-dichloroethane for the dip coating as follows.

A platinum wire (diameter, 0.5 mm) sealed into the end of a glass tube (diameter, 5 mm) to leave 1 cm of the wire exposed was dipped into 1,2-dichloroethane solution of **1** (1 wt%) and dried to give a polymer-coated electrode. The electrode was then treated for 12 h with a ternary solvent of water, methanol and 2-propanol (1:2:2 v/v) containing 0.5 wt% KOH in order to hydrolyse the methyl ester group of the polypeptide to the carboxylate group.

Electrochemical measurements

Cyclic voltammetric measurements were performed at 25°C using a Solartron Co. model 1286 potentiostat with a conventional design for a three-electrode system. A platinum plate (10 × 10 mm) and a standard Ag/AgCl (saturated KCl) electrode were used as counter and reference electrodes, respectively.

FT*i.r. measurements*

The polymer film was formed on a platinum mesh (10 × 10 mm, 60 mesh) by dip coating and treated similarly to that on the electrode. Transmission spectra of the treated film were taken on a Nicolet 510M Fourier transform spectrophotometer. The i.r. spectra were measured at 2 cm^{-1} resolution and signal-averaged over 1024 scans.

RESULTS AND DISCUSSION

Cyclic voltammograms of the ferrocyanide/ferricyanide redox couple with the modified electrode are shown in *Figure 2*. The peak currents due to the reversible electrode reaction of a $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ system on a bare platinum electrode were almost completely suppressed by coating with **1**. This indicates that the electrode was covered with hydrophobic polymer and was insulated from redox-active species.

When the polymer-coated electrode was treated for 12 h with a ternary solvent of water, methanol and 2-propanol (1:2:2 v/v) containing 0.5 wt% KOH, the cyclic voltammogram peaks appeared at almost the same potential as, but with much smaller current values than, those of the bare electrode (*Figure 2*). Alkaline treatment was carried out in order to hydrolyse the methyl ester group of the polypeptide to the carboxylate group. Thus, on hydrolysis, the polypeptide segment of **1** would be converted to the hydrophilic poly(L-glutamate), which should allow the indicator ions to penetrate into the polymer layer.

Detailed characterization of the modified electrode was not carried out. It seems rational, however, to assume a microdomain structure: polypeptide microdomains are considered to be formed in the matrix from vinyl polymer chains on the electrode, since multiphase materials including block and graft copolymers have been known in general to form microdomain structures in the solid state[8]. In fact, some of those containing polypeptide segments were found to have microdomains composed of polypeptide[8–12]. In the present case, the segments from the polypeptide were considered to form hydrophilic microdomains after hydrolysis (*Figure 3*). The microdomains containing flexible polyanionic chains would function as ion channels which allow the electrochemical communication of the redox species with the electrode, so that the cyclic voltammogram peaks reappear. The electrode surface should be, however, still covered to

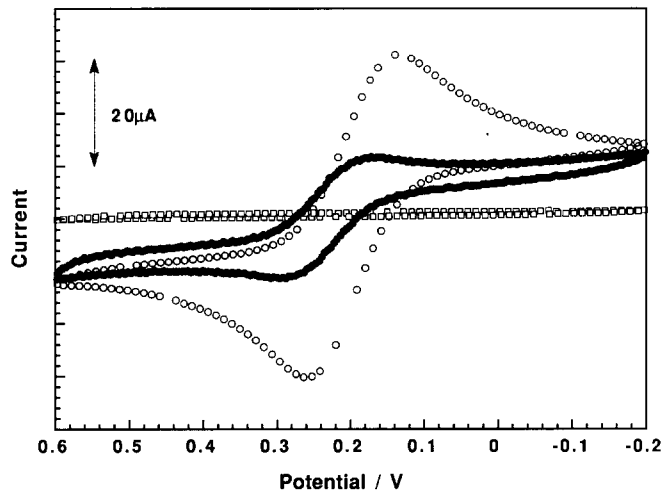

Figure 2 Cyclic voltammetric responses of the modified electrode: (○) bare platinum electrode, (□) platinum electrode coated with **1**, and (●) the modified electrode (after hydrolysis); at 25°C; scan rate, 25 mV s^{-1}; $[K_4[Fe(CN)_6]] = [K_3[Fe(CN)_6]] = 1$ mM, [KCl] = 10 mM

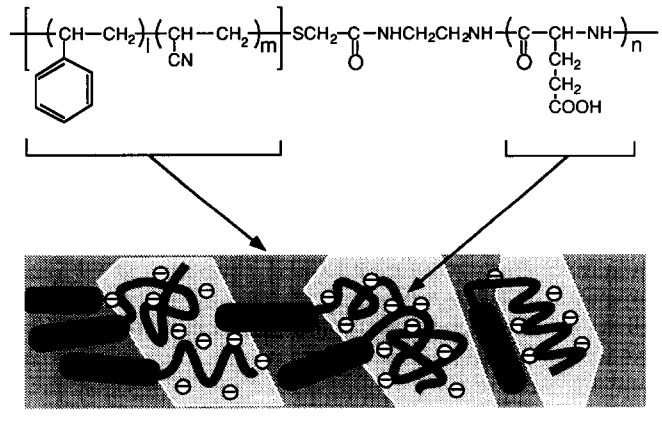

Figure 3 Schematic diagram for the biomembrane-mimetic sensor; formation of chemoreceptive, channel-like microdomains from poly(L-glutamate) chains

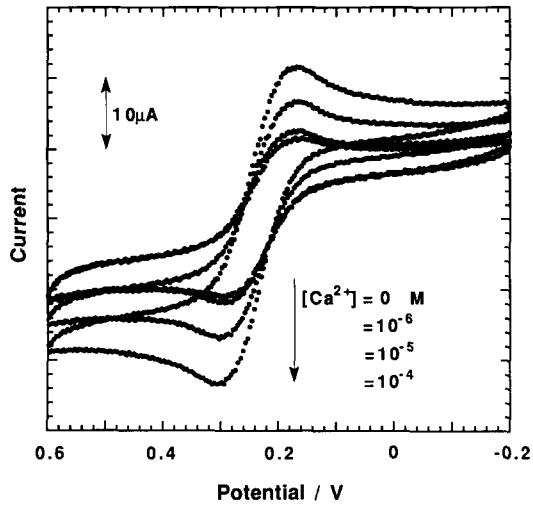

Figure 4 Effects of Ca^{2+} on current–potential curves for the **1**-modified platinum electrode; at 25°C; scan rate, 25 mV s^{-1}; $[K_4[Fe(CN)_6]] = [K_3[Fe(CN)_6]] = 1$ mM, [KCl] = 10 mM

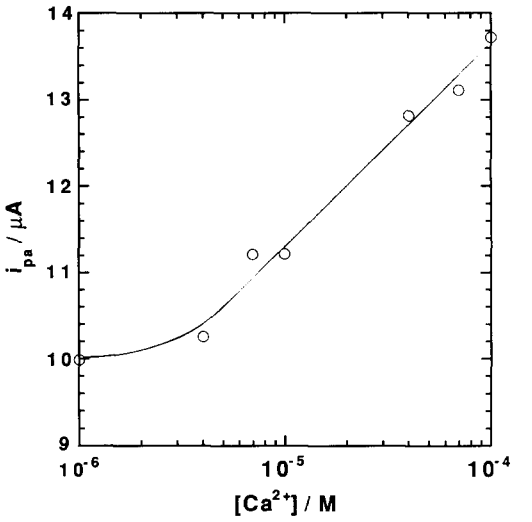

Figure 5 Ca^{2+}-dependent changes in the anodic peak current (i_{pa}) of cyclic voltammograms taken similarly to those in *Figure 4*

some extent with hydrophobic vinyl polymer segments which serve as the building blocks of the membrane. In addition, the polyanion network would reduce the local concentration of the anionic redox couple near the electrode surface, mainly due to electrostatic repulsion. These two features can account for the smaller values of peak currents than those of the bare electrode.

When adding Ca^{2+} (as $CaCl_2$), the peak currents increased significantly with increasing Ca^{2+} concentration, as seen in *Figure 4*. Please note, the sensitivity to Ca^{2+} depends upon the ionic strength of the supporting electrolyte; 10^{-4} M Ca^{2+} was the detection limit[7] when using 0.1 M KCl, whereas the sensor became susceptible to 10^{-6} M Ca^{2+} when using 10 mM KCl in these measurements. The value of the anodic peak current showed an almost linear relationship with the logarithmic concentration of Ca^{2+} in the range of 10^{-6}–10^{-4} M (*Figure 5*). Such ion concentration-dependent changes in the cyclic voltammogram profiles were also seen for Mg^{2+}, but not for Na^+, K^+ and tetrabutylammonium. With the addition of Ca^{2+}, the ionic crosslinking of carboxylate groups should occur, resulting in the contracted conformation of poly(L-glutamate) chains. The relatively open feature of the ion channels as well as the reduced number of anionic sites in the channel was considered to account for the increase in the local concentration of the redox species, leading to the enhancement of the peak currents.

Some experiments were carried out in order to confirm the above discussion for the mechanism of the electrochemical responses. The results using three types of indicators, namely anionic, neutral and cationic, are listed in *Table 1*. The value α represents a degree of suppression of the peak current by the modification based on the unmodified (bare) as 100. The anionic redox couple showed a large suppression, whereas the cationic redox couple showed quite a small suppression. Electrostatic repulsion between the polyanion on the electrode and the anionic indicator ions was thus found to be operative. On the other hand, the value β demonstrates the effect of the charge of the indicator on the response towards Ca^{2+}. Electrostatic repulsion was again seen to be a major factor; the decrease in the number of anionic sites brought about by binding with Ca^{2+} should reduce the repulsion between the modified layer and the anionic markers. The non-ionic marker, however, showed a substantial response; the mechanism cannot be explained solely by the charge. The conformational change induced by ionic crosslinking on the addition of Ca^{2+} would be an additional important factor for the sensor response.

Table 1 Effect of charge of redox-active ion as the indicator on the peak suppression in cyclic voltammogram profiles (α)[a] and the susceptibility to Ca^{2+} (β)[b]

Indicator[c]	Charge	α	β
$Fe(CN)_6^{3-}$	Negative	37.1	71.3
p-Quinone	Neutral	66.1	17.8
$Ru(bpy)_3^{2+}$	Positive	82.6	6.5

[a] $\alpha = i_{pa}$(modified) × 100/i_{pa}(bare)
[b] $\beta = \{i_{pa}([Ca^{2+}] = 10\text{ mM}) - i_{pa}([Ca^{2+}] = 0\text{ M})\} \times 100/i_{pa}([Ca^{2+}] = 0\text{ M})$; the values were determined for the modified electrode
[c] [indicator] = 5 mM, [KCl] = 0.1 M, at 25°C; bpy, bipyridine

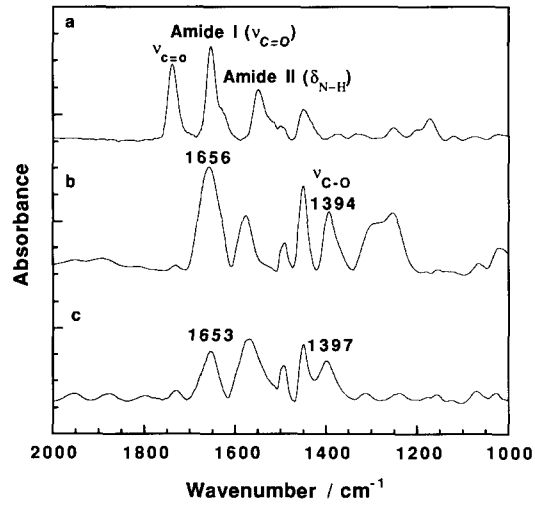

Figure 6 *FT*i.r. spectra of the polymer film from **1**: (a) the intact, (b) that treated with alkaline (KOH) solution, and (c) the hydrolysed film treated with Ca^{2+}

In order to obtain information on the structure of the polypeptide, *FT*i.r. measurements were carried out for film from **1**. *Figure 6* shows the *FT*i.r. spectra of the polymer film. The intact film showed the typical absorption for ester at 1740 cm^{-1}, which disappeared almost completely on alkaline hydrolysis. The absorption band assigned to amide I became rather broad after hydrolysis, suggesting that the polypeptide took a random coil conformation. Ca^{2+} treatment made a small change which involved a slight shift of the absorption at 1394 to 1397 cm^{-1} and a sharpening of the amide I band. The former change would be due to the binding of carboxylate with Ca^{2+}, whereas the latter may be ascribable to the conformational change of the poly(L-glutamate) chain.

It is known that a conformational change of polypeptide takes place on applying various stimuli including H^+, ions, chemical substances, solvent composition, etc. By making use of these advantages, the multiphase polymer membranes containing polypeptide would serve as a key material for the chemical modification of electrodes. In fact, the poly(L-glutamate) modified electrode has shown electrochemical responses towards urea; the concentration of urea can be determined directly, without relying on enzymes[13].

Our newly designed ion sensor based on multiphase material thus demonstrated important features of 'ion channel'-dependent biological sensing. The sensor is quite simply prepared just by dip coating and hydrolysis. In addition, the modified electrode has remarkable stability; the electrode has exhibited a response to Ca^{2+} for 2 months, when stored in 0.1 M KCl at room temperature. The facile modification, as well as the excellent stability, should be ascribed to the multiphase structure; vinyl-made membrane supporting ion channels. Synthetic polypeptides have been studied extensively as models of proteins, though their use in practical purposes has been rather limited. The strategy described in this paper would facilitate the use of synthetic polypeptides for advanced, intelligent materials.

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