Electrode Modification by Long-Chain, Dialkyl Disulfide Reagent Having Terminal Dinitrophenyl Group and Its Application to Impedimetric Immunosensors

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A long-chain, dialkyl disulfide having a terminal dinitrophenyl (DNP) group was newly synthesized for modification of a surface of gold with chemisorbed, self-assembled organic monolayers. The resulting monolayer was characterized by infrared spectroscopy of reflection absorption spectroscopy mode, by cyclic voltammetric measurement and by electrical impedance measurement. Coupled with impedance measurements, a possible application of the modified electrode system to impedimetric immunosensors was demonstrated. The sensor gave electrical response to anti-DNP antibody at $10 - 10^3$ ng cm⁻³ concentration.

Keywords Bifunctional alkanethiol, chemisorption, self-assembled monolayer, modified gold electrode, impedimetric immunosensor

Recent advances in chemically modified electrodes (CMEs) have stimulated considerable research interest in their application to electrochemical sensors.¹ Central to both the fundamental study of CMEs and their sensor applications is the rational manipulation of the electrode surface functionality by immobilizing selected types of molecules. One of the recent works in this area has focused on the monolayer formation on surfaces of gold from organosulfur precursors.²

Stable attachment of organic molecules to gold and other metal surfaces is attained through the use of reagents having thiol, sulfide, disulfide, or other sulfur-containing head groups by immersing gold substrate into their dilute solutions. In case of compounds having a long-chain alkyl group, monolayers with highly-ordered, well-defined structure are formed spontaneously. This attachment has been described as due to the formation of some type of thiolate complex on the gold surface, and therefore, the use of sulfur-containing species as anchors on gold seems to be especially attractive for CMEs' application because of the stability of the surface complex formed.

However, formation of densely-packed hydrocarbon monolayers becomes a disadvantage to electrochemical application. The redox reaction of the target analyte is blocked almost completely on the modified electrode. Use of functionalized thiols with redox-active groups³ can be one promising way to overcome this difficulty, because such modifiers can mediate an electron transfer between the gold substrate and the analyte in solution,

making the amperometric measurement feasible. There can be another sensing approach which now makes use of the electrically insulating property itself of the modified gold surface. It is an impedimetric sensing⁴, where the change in electrical impedance characteristics (change in dielectric property) of the modified surface layer is used to sense the chemisorbed species on the sensing electrode surface. In the present study, we synthesized a dialkyl disulfide compound having terminal dinitrophenyl groups (dinitrophenyl: DNP) (1) and studied some fundamental physico-chemical characteristics of the 1-modified gold electrode for its possible application to impedimetric sensing of protein (anti-DNP antibody).

Antigen and antibody measurements are important in clinical chemistry, physiology, and modern biotechnology. However, if one considers their determination by electrochemical technique, a problem arises because antibody-antigen reactions are usually not directly linked to redox processes. In addition, the reactions are stoichiometric and do not involve chemical amplification. This is in a marked contrast to enzyme reactions which often give potentiometrically or amperometrically measurable reaction products in an amplified manner. Reflecting these situations, considerable efforts have been made to date to develop electrochemical immunosensing devices such as electrochemical enzyme immunoassay⁵, potentiometric ionophore-modulation immunoassay⁶, and piezoelectric systems.⁷

On the other hand, a different approach has recently been made to detect the antibody-antigen reactions

electrochemically. An immunochemical reaction is carried out on an electrode surface and the change in dielectric property of the surface layer is measured. The effort is to exploit capacitive or impedimetric immunosensors. Martelet *et al.* reported quite simple, direct detection of immunospecies by capacitance measurements on a silicon/silicon dioxide/immobilized antibody/electrolyte heterostructure.⁸ They considered the electrode surface as having an ideally blocked layer structure, each layer carrying a specific electric capacitance in a series of combinations. In order for this type sensory technique to have generality, it seems important that the quality of such surface layer properties as structural homogeneity and integrity are controlled reproducibly in the electrode fabrication process. Further, it is emphasized that one may be able to evaluate a.c. parameters of the fabricated layer properly only when the nature of the electrical equivalent circuit is reasonably understood on the chemical basis on the electrode.⁴

We supposed that such factors in electrode fabrication as pointed out above are most conveniently studied on a

gold electrode by thiol modification technique. In the present study, we synthesized a dialkyl disulfide compound carrying a terminal dinitrophenyl group (**1**). An organic monolayer therefrom was developed on the surface of the gold substrate and was characterized by Fourier-transform infrared spectroscopy (FT-IR) and electrochemical methods. A possible application to an electrochemical immunosensor based on an impedimetric principle was also investigated.

Experimental

Chemical and immunochemical reagents

Chemicals for synthetic use were of extra pure grade. All other chemicals were guaranteed reagents and were used without further purification. Immunochemical reagents, monoclonal anti-DNP antibody (Sigma, from mouse sources: subclass, IgE) and monoclonal anti-IgE antibody (from rat sources, Cosmo Bio Co.) were used as received.

Reagent **1** was prepared by a synthetic reaction in Scheme 1.

N-(6-Aminohexyl)-2,4-dinitroaniline

1-Chloro-2,4-dinitrobenzene was reacted with a slight molar excess of 1,6-diaminohexane in dichloromethane for 24 h at room temperature.⁹ After evaporation of the solvent and the excess 1,6-diaminohexane under reduced pressure, the residue was subjected to silica gel column chromatography to obtain *N*-(6-aminohexyl)-2,4-dinitroaniline.

11,11'-Dithiodiundecanoic acid

11-Bromoundecanoic acid was allowed to react with an equimolar amount of thiourea in ethanol to give a thiuronium salt, which was then hydrolyzed with aqueous potassium hydroxide to give 11-mercaptoundecanoic acid.¹⁰ The thiol obtained was then oxidized with hydrogen peroxide¹¹ to give the disulfide.

Bis(10-(*N*-(6-(2,4-dinitrophenylamino)hexyl)carbamoyl)decyl)disulfide(**1**)

While cooling externally in an ice-bath, 11,11'-dithiodiundecanoic acid (0.15 g) was treated with *N*-(6-aminohexyl)-2,4-dinitroaniline (0.2 g) in 20 cm³ dichloromethane in the presence of *N,N*-dicyclohexylcarbodiimide

Fig. 1 Structure of **1** and schematic illustration of chemisorbed **1**-monolayer on gold surface.

Scheme 1.

(0.15 g) for 24 h. After evaporation of the solvent, the residue was subjected to column chromatography (silica gel) to obtain **1**: yellow powder; mp 89.5–90°C. ^1H -nmr (HITACHI, Ltd., R-24B) (60 MHz, CDCl_3 ; δ ppm downfield of tetramethylsilane) 1.42 (m, 28H, CH_2), 2.17 (m, 3H, $\text{CH}_2\text{-CO}$), 2.64 (t, $J=7$ Hz, 2H, S-CH_2), 3.32 (m, 4H, NH-CH_2), 6.05 (t, $J=6$ Hz, 1H, CO-NH), 6.91 (d, $J=9.6$ Hz, 1H, C-6 CH), 8.19 (dd, $J=2.8, 9.6$ Hz, 1H, C-3 CH), 8.51 (t, $J=5$ Hz, 1H, $\text{NH-C}_6\text{H}_3(\text{NO}_2)_2$), 9.00 (d, $J=2.8$ Hz, 1H, C-3, CH). IR (KBr), 2925, 2851, 1630, 1588, 1542, 1527, 1422, and 1352 cm^{-1} . Elemental analysis: calculated for $\text{C}_{46}\text{H}_{74}\text{O}_{10}\text{N}_8\text{S}_2$ (found); C, 57.35% (56.91%); H, 7.74 (7.74); N, 11.64 (11.64).

FT-IR measurement

IR spectral measurements, both transmission mode in KBr pellet and RAS (reflection absorption spectroscopy) mode, were performed on a Nicolet 510M spectrometer equipped with a MCT detector. The transmittance spectrum was measured at a spectral resolution of 4 cm^{-1} with 10 scans. For RAS mode, gold substrates were first prepared by vacuum evaporation onto slide glasses. A monolayer of **1** was prepared by immersing a freshly prepared substrate into a 1,4-dioxane solution of **1** (concentration, 10 mM) for 24 h at room temperature. The substrate was carefully rinsed with 1,4-dioxane, dried under vacuum and was used for measurements immediately. The p -polarized radiation was introduced on the sample at 85° off the surface normal and data were collected at a spectral resolution of 4 cm^{-1} with 1095 scans.

Electrochemical measurements

A gold disk electrode (diameter 1.6 mm, Bioanalytical Systems Co.) was used as working electrode through out the present electrochemical measurements. The electrode surface was polished to a mirror finish with alumina powders of successively finer grades (Buehler Co.) and was chemically modified with the monolayer of **1** by the same procedure as described above.

Cyclic voltammetric measurements were performed by using a Solartron Co. Model 1286 potentiostat with a design of a three-electrode system. A Pt plate (10×10 mm) and a standard Ag/AgCl (saturated KCl) electrodes were used as counter and reference electrodes, respectively.

The apparatus used for impedance measurements¹² was composed of a Solartron Co. Model 1250A frequency response analyzer coupled with a Model 1286 potentiostat. A three-electrode configuration using a counter electrode (Pt plate, 10×10 mm) and a standard Ag/AgCl reference electrodes (saturated KCl) was employed for impedance measurements.

Frequency response analysis (FRA) involves a study of the way in which the total impedance of the cell varies with frequency. The working electrode is held at a constant potential with respect to a reference electrode, and a superimposed overlapping small perturbation voltage is applied between the working electrode and the

counter electrode. The electrical response of the circuit is then measured and analyzed. In practice, an open-circuit potential of the working electrode was first determined before each FRA measurement. The open-circuit potential was monitored against a reference electrode under nitrogen until it became constant (typically 30 min). The potential of the working electrode was then held at this value, and a 50 mV (rms) sinusoidal wave ranging from 0.1 Hz to 65 kHz was applied. All experiments were made at a constant temperature of $25\pm0.2^\circ\text{C}$.

Results and Discussion

Synthesis of immuno-affinity anchoring reagent **1**

In designing the reagent **1**, we took the binding affinity between the antibody and a series of hapten molecules into consideration. Herman *et al.*¹³ investigated the affinities of anti-2,4-DNP antibodies to a variety of 2,4-dinitrobenzene derivatives and found ϵ -N-DNP-L-lysine to have the highest affinity to the antibody. Because antigens typically have a molecular weight greater than 1500 to elicit efficient antibody formation, antibodies to small molecules, such as DNP moiety, are obtained by chemically attaching the molecule to some larger carrier antigen, usually a protein. Such chemical modifications of proteins, in general, are suggested to occur at an ϵ -amino group of lysine. This explains the high antibody affinity of ϵ -DNP-L-lysine. Based on this consideration, we designed the immuno-affinity anchoring reagent **1** which incorporated N -alkyl-2,4-dinitroaniline structure.

Characterization of organic monolayer on gold by IR spectroscopy

Figure 2 shows IR spectra found for **1** in monolayer state on gold substrate and in microcrystalline state in a KBr disk. The RA spectrum of the **1**-treated gold substrate shows fundamentally a spectrum similar to the transmittance spectrum of microcrystalline **1**. In the high-frequency region, RA spectrum shows a broad peak around 3300 cm^{-1} which can be assigned to overlapped N-H stretching modes of the amino and amide groups. In the transmittance spectrum in KBr disk, this band is further obscured by a broad O-H stretching band due to the adsorbed moisture. Two peaks at 2933 cm^{-1} and 2860 cm^{-1} can be identified as C-H stretches for anti-symmetrical and symmetrical modes of the connecting methylene group, respectively. In the mid-frequency region, which contains most of the information regarding the structural feature of **1**, an intense peak at 1629 cm^{-1} can be assigned to C=O stretch mode of amide group. Two peaks at 1593 and 1344 cm^{-1} are NO_2 stretches of antisymmetrical and symmetrical modes, respectively. A broad band at 1427 cm^{-1} can be identified as CH_2 scissoring mode.

The positions of peak frequencies of $\nu(\text{CH}_2)$ modes give insight into the "crystalline-like" or "liquid-like" nature of the monolayer.¹⁵ From the literature¹⁴, the

Fig. 2 FT-IR spectra in (a) high-frequency region and in (b) mid-frequency region. RAS denotes reflection absorption spectra of **1**-treated gold substrate and TS denotes transmittance spectra of microcrystals of **1** dispersed in a KBr disk.

peak position of the $\nu_{as}(\text{CH}_2)$ mode of crystalline polymethylene chain (2920 cm^{-1}) is 8 cm^{-1} lower than that for the liquid state (2928 cm^{-1}), whereas for $\nu_s(\text{CH}_2)$ mode the peak position is 6 cm^{-1} lower in the crystalline state (2850 cm^{-1}) than that in the liquid (2856 cm^{-1}). Compound **1** contains two different methylene chains. Thus, some ambiguities are introduced into the interpretation of the CH_2 -stretches. However, the peak positions of $\nu_{as}(\text{CH}_2)$ mode and $\nu_s(\text{CH}_2)$ mode for the monolayer **1** are 8 cm^{-1} and 9 cm^{-1} higher than those for microcrystalline **1**, respectively. These data suggest that **1** forms "liquid-like" monolayers on gold surface, in contrast to those monolayers from simple alkanethiols with long alkyl chain.¹⁵ Based on Corey-Pauling-Koltun (CPK) model building, the cross section of the DNP group was estimated to be $38\text{ \AA}^2$ ($8\text{ \AA} \times 4.8\text{ \AA}$), while it was only $14\text{ \AA}^2$ ($3.8\text{ \AA} \times 3.6\text{ \AA}$) for polymethylene chain. This incompatibility in molecular dimension along the terminally

dinitrophenyl-substituted alkyl chain should lead to the "liquid-like" nature of the polymethylene chain in the monolayer formed from **1**, since the placement of a bulky DNP group upon the alkyl chain layer inevitably precludes the closest packing of polymethylene chains.

Anyhow, the results obtained above strongly suggest that **1** also adsorbs onto gold surfaces in a similar manner to simple alkanethiols. Consequently, we are now able to modify gold surfaces with an organic monolayer film having dinitrophenyl terminal groups.

Characterization of monolayer by cyclic voltammetry

In order to probe the adsorption behavior of **1** and the nature of the resulting monolayer, we studied **1**-modified gold electrode by cyclic voltammetric and impedance measurements. The former technique gave information on the extent of monolayer coverage on the electrode surface, while the latter gave insight into physicochemical nature of the monolayer.

Figure 3 shows cyclic voltammograms measured on **1**-modified gold electrode in $0.1\text{ M H}_2\text{SO}_4$ solution. Under such strong acidic conditions, the monolayers were expected to deteriorate rapidly, but the cyclic voltammograms were not affected on the repeated potential scanning. The gold oxide removal peak at around 1.0 V was integrated by a graphical procedure, and the integrated value was compared with the corresponding value from the untreated bare gold electrode to estimate the surface coverage ratio, θ ($\theta=0$ for bare electrode; $\theta=1$ for completely covered electrode).¹⁶ To confirm the reproducibility of surface modification and θ measurement, the same gold electrode was repolished, surface-modified (treatment with **1**), and subjected to the same voltammetric study. Such repeated θ measurements gave θ values of 0.14 , 0.19 , 0.31 for three independent runs. For comparison, dodecanethiol was also used as electrode modifier and a

Fig. 3 Cyclic voltammograms for (a) bare gold electrode, (b) the gold electrode after treatment with **1** (the voltammogram of its unmodified state is (a)) and (c) $\text{C}_{12}\text{H}_{25}\text{SH}$ -modified electrode (the voltammogram of its unmodified state is not shown). Electrolyte solution, aqueous $0.1\text{ M H}_2\text{SO}_4$; scan rate, 100 mV s^{-1} ; temperature, 25°C .

θ value of 0.85 was obtained.

Surface coverage on gold substrate should suffer such experimental uncertainties as the roughness of (polished) gold surface, the extent of activation of Au surface (cleanness of surface), and so on. Taking these factors into consideration, the reproducibility of surface coverage indicated above seems reasonably good. On the other hand, the low θ value for **1** relative to that of dodecanethiol monolayer may be ascribed to the steric hindrance due to the bulky DNP group. As already noted before, the cross section of the DNP group is estimated to be $38 \text{ \AA}^2$ while it is only $14 \text{ \AA}^2$ for polymethylene chain. This means that the effective (greatest) molecular cross section of **1** is 2.7 times larger than that of dodecanethiol. If one makes a simple assumption that θ value is reciprocally proportional to the greatest cross section within the molecular chain, the θ value expected for monolayer of **1** would be $1/2.7$ times that observed ($\theta=0.85$) for dodecanethiol monolayer, that is, $0.85 \times (1/2.7) = 0.32$. The experimental value agrees fairly well with this estimation.

Electron transfer measurements were then made in aqueous solution by using cyclic voltammetric techniques. A $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple was used since it represents an electrochemically reversible, well-defined, one-electron redox reaction with moderate normal potential ($E^\circ=0.21 \text{ V}$). Figure 4 shows typical cyclic voltammograms obtained with a bare gold electrode and a **1**-modified gold electrode having a θ value of 0.31. Although a small current due to ferrocyanide oxidation appears with a large overpotential, most of the current observed at the modified electrode is capacitive. Therefore, the monolayer of **1** on the gold electrode effectively blocks the electron transfer process to and from the solution species in spite of its low surface coverage ratio. This blocking effect may be partially due to the hydrophobic character of the monolayer.

Fig. 4 Cyclic voltammograms for (a) bare Au electrode and (b) **1**-modified gold electrode. Electrolyte solution: aqueous 0.1 M KCl containing 5 mM each of $\text{K}_4[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$. Scan rate, 50 mV s^{-1} ; temperature, 25°C .

Characterization of monolayer by impedance spectroscopy

In the presence of redox-active species in solution, an impedance response of the electrode-solution system is conveniently expressed in terms of Randles' equivalent circuit. The circuit is illustrated below:

where R_{soln} , C_{dl} , R_{CT} and Z_{W} represent a resistance of the electrolyte solution, a capacitance of the electrical double layer on the electrode surface, a resistance to redox (charge transfer) reaction at the electrode surface and a Warburg impedance, respectively. The Warburg impedance represents a resistance when redox-active species diffuse through the boundary layer on the electrode surface. In Randles' equivalent circuit, Faradaic impedance (an electrical impedance which accompanies Faradaic current) is represented by a serial combination of R_{CT} and Z_{W} .

Figure 5 shows complex impedance diagrams obtained in our system for bare and modified electrodes in the presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple. For ordinary redox processes on metal electrodes, Randles' equivalent circuit gives a complex impedance diagram which shows the so-called kinetic semi-circle in a high frequency region, since the diffusion of redox species does not contribute much to the total impedance and the

Fig. 5 Complex impedance plots for (a) bare gold electrode and (b) **1**-modified gold electrode having θ value of 0.26. Electrolyte solution: aqueous 0.1 M KCl containing 5 mM each of $\text{K}_4[\text{Fe}(\text{CN})_6]$ and $\text{K}_3[\text{Fe}(\text{CN})_6]$; temperature, 25°C . Measurements were made at 50 mV (rms) around E' of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ couple (typically, $+209$ – $+217 \text{ mV}$). Solid lines show theoretical calculation according to Eq. (1) based on curve fitting to experimental data.

equivalent circuit is reduced to a parallel combination of C_{dl} and R_{CT} (plus serial R_{soln}). In a low frequency region on the other hand, the impedance is dominated by a diffusion of redox species and the equivalent circuit reduces simply to a serial combination of R_{soln} and Z_w , which now gives a 45° straight line in the diagram. The whole impedance diagram therefore gives a semi-circle fused with a 45° "diffusion" line. In Fig. 5, the bare gold electrode exactly shows these features. This means that Randles' equivalent circuit is properly applicable to this electrochemical system.

In case of the modified electrode, only a kinetic semi-circle was observed, and a 45° diffusion line at low frequencies did not show up clearly in the frequency range studied. The electrical parameters which determine the pattern of impedance diagram are R_{CT} and its relative magnitude to that of Warburg impedance. In case of the electrochemical system having a large R_{CT} (i.e., a charge transfer process is kinetically rather sluggish), the diagram shows a 45° diffusion region only partially, because mass transfer is not a significant factor in most of the frequency range. As already noted, surface monolayer of **1** significantly blocks the electron transfer process at the electrode-solution interface. This should lead to a large R_{CT} value of the system, which in turn means an enlarged semicircle in the impedance diagram, leading to the lack of a clear 45° linear portion in the plots. On the other hand, contrary to our first expectations, the monolayer of **1** did not totally block the redox reaction by the solution species. This may be due to the low θ value of the electrode. However, it should be emphasized that the impedance diagram itself is clear and reproducible enough to evaluate the a.c. parameters by adopting the Randles' equivalent circuit.

First, R_{CT} values were obtained by non-linear curve fitting procedure from the experimental impedance data obtained in the frequency region above 8 Hz. In this frequency region, the impedance plots can be safely regarded as forming a part of a semi-circle. The following equation was used in the curve fitting calculation:

$$I_m = (- (R_e - l)^2 + m^2)^{1/2} + n \quad (1)$$

where R_e and I_m stands for the real and imaginary parts of the impedance, respectively. The parameters l and n stand for the coordinate of the center of the circle, and m stands for the radius of the circle. The R_{CT} values estimated were in the range 0.3 – 0.5 MΩ cm⁻² and 0.6 – 1.8 MΩ cm⁻² for bare and **1**-modified electrodes, respectively, for several independent electrode preparations. The electrical capacitance C_{exp} can be evaluated from the frequency f_{max} , which represents the upper maximum point of the semi-circle in the complex impedance plots, according to the relationship¹⁸

$$\begin{aligned} \omega_{max} &= 1 / (R_{CT} C_{exp}) \\ &= 2\pi f_{max}. \end{aligned}$$

It seems reasonable to make a simple approximation that C_{exp} is a parallel combination of the double-layer capacitance C_{dl} at the unmodified, bare gold surface and the modified-layer capacitance C_m .¹⁶ This gives the expression

$$C_m = [C_{exp} - C_{dl}(1 - \theta)] / \theta.$$

The dielectric constant ϵ of the modified layer (monolayer of **1**) can then be calculated by assuming the monolayer thickness.

Unfortunately, the C_{dl} value of the bare gold electrode was found to be sensitive to such experimental uncertainties as the mean surface area (roughness of the electrode surface) and adsorbed impurities. Thus, we only obtained rather scattered values for C_{dl} ranging from 17 to 22 μF cm⁻². For the modified electrode having θ value of 0.31; the C_{exp} value obtained was 17 μF cm⁻². By assuming the thickness of 3.3 nm for the monolayer, which was estimated by CPK model building, the effective dielectric constant of the monolayer ranging from 22 to 57 μF cm⁻² was calculated. The dielectric constant of simple alkanethiols aligned in the monolayer on Au surface is reported to be in the range 2 – 3 μF cm⁻²; at first glance, the data obtained above in the present study look unusual. However, the dielectric constant of liquid nitrobenzene is 34.6 μF cm⁻². In addition, as noted in the previous section, the monolayer of **1** was found to possess "liquid-like" nature on gold surface. If we consider these points, the relatively large monolayer dielectric constant obtained above might not be so unusual.

Impedance measurements were also made in the absence of [Fe(CN)₆]^{3-/4-} redox-couple in solution phase. Under such experimental conditions, the open-circuit potential can not be defined and can change. However, experimentally, the electrode potential measured under nitrogen did not show much change in the course of ordinary impedance measurement, staying typically between +0.45 and +0.49 V (vs. Ag/AgCl). A typical example of impedance diagram obtained under such conditions is shown in Fig. 6.

The profile at the 65 kHz – 6.3 Hz region is similar to that measured in the presence of redox couple. The profile in this frequency region was quite reproducible at measurements with independent electrode preparations. However, at 5 Hz and below, the data points began to scatter and the reproducibility of the measurement was lost. This seems inevitable since the system lacks redox species that are to carry out a net electron transfer reaction between the electrode and the solution phase. However, by using the data in the region above 6.3 Hz and by assuming the equivalent circuit similar to that adopted in Fig. 5, R_{CT} values ranging from 20 to 30 MΩ cm⁻² were calculated by a curve fitting procedure (Eq. (1)); these were one order larger than the R_{CT} values obtained in the presence of [Fe(CN)₆]^{3-/4-} couple. This R_{CT} value also led to the C_{exp} values (0.5 μF cm⁻²) which were in fair agreement with those values (0.2 –

Fig. 6 Complex impedance plots for 1-modified gold electrode in the absence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple. (a) Impedance diagram in the whole frequency region studied. ●, 65 kHz–6.3 Hz; ▲, 5.0 Hz–1.3 Hz; ■, 1.3 Hz–0.1 Hz. (b) Impedance diagram in high-frequency region (enlarged). ●, 65 kHz–6.3 Hz; ▲, 5.0 Hz–1.3 Hz. Electrolyte solution: aqueous 0.5 M KCl containing 0.05 M each of boric acid–NaOH buffer (pH 8.0); temperature, 25°C. Broken lines show theoretical calculation based on curve fitting to experimental data at 65 kHz–6.3 Hz.

0.6 $\mu\text{F cm}^{-2}$) obtained in the presence of redox couple.

From a strict electrochemical viewpoint, measurements in the absence of redox couple are not adequate to study the precise nature of a chemically modified gold electrode. However, as shown in the observations above, impedance behavior of the modified electrode does not suffer from modification in the absence of redox couple if the measurements are limited in the high frequency region (≥ 6.3 Hz). Therefore, we decided to employ a redox couple-free system to explore the possibility of impedimetric immunosensing. The absence of redox couple is also expected, at the first approximation, to diminish chemical complexity of the system when the adsorption of a protein (anti-DNP antibody) is to be studied on the electrode surface.

Application to impedimetric immunosensor

We have described above the preparation and the characterization of 1-modified gold electrode. In the same way as in the previous section, FRA study was made for the 1-modified gold electrode in the presence of antibodies. The impedance diagram obtained was fundamentally similar to that in Fig. 6 (which was obtained without antibodies), but the plots shifted slightly upward. Because of the same difficulty as in Fig. 6, the intrinsic value of electrical capacitance of the modified layer itself could not be determined precisely. Further, an additional difficulty arose since the immuno-reaction on the electrode surface influenced not only C_m but also R_{CT} values. A detailed discussion is beyond the scope of the present study. These circumstances led us to discuss the sensory application conveniently by using a combined electrical impedance which includes both C_m and R_{CT} .

The electrical impedance of the 1-modified electrode was measured at 100 Hz while changing the concentration of antibody in the solution. The impedance was first measured in the absence of antibody, and the change or increment on the succeeding addition of the antibody was recorded. The results are summarized in Fig. 7. In the case of monoclonal anti-DNP antibody, the electrical impedance increased as the concentration of the antibody increased, while such concentration dependence was not observed in case of the addition of monoclonal anti-IgE antibody. These observations strongly suggest that the experimentally obtained antibody response arises from the binding of the specific antibody protein to the corresponding antigen portion available on the modified electrode surface. The impedance response is clear at the antibody concentration as low as 10 ng cm^{-3} , but non-specific binding of anti-IgE antibody can not be negligible under similar concentration levels. The detection limit of 10 ng cm^{-3} is slightly less than that of the conventional immunoassays based on potentiometric ionophore-modulation⁶ and a piezoelectric⁷ principle.

Since the antibody molecules are electrically insulating, a piling-up of such molecules on the modified electrode surface is reasonably understood to decrease the electrochemical conductivity across the electrode/modified layer/solution interface. Unfortunately, it is impossible at present to experimentally evaluate the effect of such antibody binding on the C_{dl} value. However, we can expect that the binding of nonpolar, organic molecules on an electrode surface reduces the C_{dl} value.¹⁹

Although we have demonstrated positively the possibility of impedimetric immunosensing by using a

Fig. 7 Electrical impedance response of modified electrode to (●) anti-DNP antibody and (○) anti-IgE antibody. Frequency response analyses were made at 50 mV (rms) around the natural potential (under nitrogen typically, +450 – +490 mV), and the change in electrical impedance at 100 Hz was plotted against the antibody concentration. Bars in the figure show variations in the response when the measurements were made with the two separate preparations of the electrode. Electrolyte solution: aqueous 0.5 M KCl containing 0.05 M each of boric acid–NaOH buffer (pH 8.0); temperature, 25°C.

modified gold electrode, the results presented here need to be supplemented with further investigations. One of the major problems is the variation of impedance according to the preparations of the modified electrode. For example, one preparation of 1-modified electrode showed an impedance value of 7.5 kΩ at 100 Hz in the absence of antibody. The electrode was repolished and remodified with 1 according to the standard procedure. The electrode thus obtained now showed the impedance value of 11 kΩ. This variation clearly indicates a poor reproducibility of the roughness and the chemical reactivity of the polished gold surface. However, it is important that the response to the antibody concentration remained within a reasonably narrow variation range, as shown in Fig. 7 despite of this large absolute impedance difference in the independent preparations of the 1-modified electrodes.

The second problem is the possible interference caused by the adsorption of other proteins such as albumin, since the formed layer of proteins due to nonspecific adsorption other than immunochemical binding should also change the electrochemical impedance of the modified electrode. Therefore, influence of co-existing serum proteins on impedimetric response should be investigated before practical application of the electrode

to blood samples. The development of biocompatible material on which protein adsorptions are prevented²⁰ would hopefully give hints to overcome this problem.

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