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Potentiometric and Spectroscopic Investigations of the Reaction of Fluorescein Isothiocyanate with an Amine Chemically Bound on Solid Surfaces

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Titanium electrode surface was chemically modified with an aminosilane. The potential of the electrode was shifted to the negative direction by a reaction of fluorescein isothiocyanate in solution with the amine on the electrode surface. The absorption and emission spectra were measured for the fluorescein isothiocyanate, fixed by the reaction with the aminosilane which was chemically bound on the surfaces of tin oxide, titanium oxide and quartz, in solutions and in air. In an alkaline solution, the absorption and emission spectra showed the band ascribable to the dianion of the fluorescein. A peak, attributable to the monoanion, was observed in both absorption and emission spectra measured for the samples kept in air. The surface coverage of the dye was estimated from the absorbances to be about 10^{-6} mol m^{-2} for both tin oxide and quartz. The bound dye fluoresced under exposure to ultraviolet light, due probably to the energy transfer from tin oxide to the dye.

Recently, immobilization techniques of organic molecules on inorganic substrates have been extensively developed by several groups in relation to electrochemical studies.[1–4]

In previous papers, we have described the electrode potential changes arising from antigen-antibody and enzyme-enzyme inhibitor reactions which take place on the electrode surface and proposed an electrical double layer model for the interpretation of these potential changes.[5–7] The applicability of such a potentiometric method to the analysis of very small quantities of biological substances in solutions has also been pointed out.

Numerous studies on surface potentials have so far been made for liquid-gas and solid-gas interfaces, but few potentiometric investigations have been made for solid-liquid interface.[8] The adsorptions of ions and molecules on the electrode surface are known to influence the electrode potential in solution. In cases where the solid surface has an active site interacting only with a particular substance, as in the case of antigen-antibody reactions mentioned above, the surface potential of the solid may change specifically by the interaction. In order to elucidate the relationship between the molecular interaction at an electrode surface and the resultant potential change, the surface reaction of an amine chemically bonded on inorganic substrates with fluorescein isothiocyanate was studied by both potentiometric and spectroscopic methods. The related physicochemical properties of solid surfaces have also been investigated.

Experimental

Materials. (3-Aminopropyl)triethoxysilane (KBE903) and methyltrimethoxysilane (KBM13) from Shinetsu Chemical Co. were used without further purification. Special grade toluene was shaken with concentrated sulfuric acid, dried over anhydrous calcium chloride for 1 d, and distilled. Fluorescein isothiocyanate (isothiocyanatofluorescein) isomer 1, abbreviated as FITC, from Sigma Chemical Co. and fluorescein from Wako Pure Chemical Industries Ltd. were used without further purification.

Preparation of Modified Titanium Electrode. The tip of a titanium wire was heated at about 1000 °C so as to produce thin oxide film on the surface. Silanization of the heat-treated tip was carried out by a method based on Murray's[9,10] as follows. The titanium wire was allowed to stand in an oven at 200 °C for 1 h, the tip was immersed into a 10% toluene solution of (3-aminopropyl)triethoxysilane and the solution was refluxed for 17 h. The tip thus aminosilanized was washed successively with toluene, acetone and distilled water, and attached to a glass tube with epoxy resin as illustrated in Fig. 1a. The electrode thus formed was heated at 80 °C for 1 h to harden the epoxy resin.

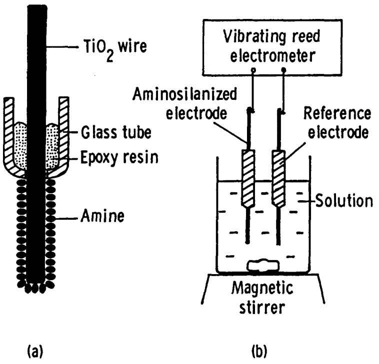

Fig. 1. Aminosilanized titanium electrode (a), and the apparatus for measuring the electrode potential (b).

The reference electrode was similarly prepared by treating the heat-treated wire with a 10% toluene solution of methyltrimethoxysilane for 17 h at 20 °C.

Silanization and FITC Modification of Tin Oxide Film, Quartz and Titanium Oxide Powder. Glass slides coated with tin oxide film were obtained from Hiraoka Glass Co. The tin oxide surface was wiped with a piece of soft cloth damped with commercially available anionic detergent. The glass was then successively washed in an ultrasonic cleaning bath with the washing agent solution for 30 min, distilled water for 10 min, isopropyl alcohol for 10 min and distilled water for 30 min, and soaked in 10 M (1 M = 1 mol dm^{-3}) sodium hydroxide solution for 17 h following the method used by Kuwana *et al.*[11] Washed with distilled water in the ultrasonic bath, the glass was dried in an oven for 1 h at 150 °C. Chemi-

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cal modification of the tin oxide film was performed by the same method as that used for the preparation of titanium electrode.

In order to prepare the FITC bound tin oxide for the spectroscopic measurements, the tin oxide film modified with the aminosilane was allowed to react with 4.4×10^{-4} M FITC solution of 0.05 M carbonate–hydrogencarbonate buffer, pH 10.4, at 5 °C overnight. An ES grade quartz plate from Japan Quartz Co. was modified according to the same method as that for the tin oxide film described above.

The coupling reaction of (3-aminopropyl)triethoxysilane to titanium oxide (rutile) powder, purchased from Merck Co., was performed in the same manner. The aminosilanized powder and *ca.* 2 mg of FITC were added at once to 30 ml of a 0.05 M carbonate–hydrogencarbonate buffer solution, pH 10.4, cooled at 4 °C to prevent the degradation of FITC and allowed to stand overnight. The modified powder was then washed with distilled water in an ultrasonic bath for 10 min and spread on a piece of filter paper and dried for 4 d.

Measurement of the Electrode Potential. The electrical potential between the modified and the reference electrodes was measured in a 30 ml reaction vessel at 20 °C by using a Takedariken TR 84M vibrating reed electrometer in the same manner as described elsewhere (Fig. 1b).[5,6]

Spectroscopic Measurement. The absorption spectra of the modified tin oxide film and quartz plate in air were measured by use of a single beam UV-visible spectrophotometer equipped with a photon counting system.[12] A Shimadzu MPS-50L UV-visible spectrophotometer was used to measure the spectra of the powder and the samples in solution.

The emission spectra were measured by using two Nippon Jarrell–Ash JE 25 monochromators having a focal length of 25 cm, a Hamamatsu R136 photomultiplier and an NF LI 573 lock-in amplifier together with a PAR 125A light chopper. The samples were excited with light of 465 nm in wavelength incident at an angle of 75° with the line normal to the sample face. The emission spectra were not corrected for the response of the monitring system.

Results and Discussion

Reaction with FITC. Figure 2 shows the change of potential with time in the aminosilanized titanium electrode by reaction with FITC. When the solution was replaced with a simple buffer solution (containing no FITC) at a time designated by X in curve a in Fig. 2, the potential held the same value as that before the replacement. The magnitude of change in potential was roughly exponential suggesting the first order kinetics of the reaction between the electrode and FITC. This kinetic result is reasonable because the number of FITC molecules in the solution is remarkably larger than that of the amino groups on the electrode surface. The concentration of FITC in the vessel was 2.1×10^{-4} M for curve a and 7.0×10^{-5} M for curve b in Fig. 2. The rate of potential change was nearly proportional to the FITC concentration. Zanker and Peter have shown that fluorescein exists as a dianion in an alkaline solution of pH>7.[13] The negative potential change observed can be explained by the attachment of dianion on the electrode surface.

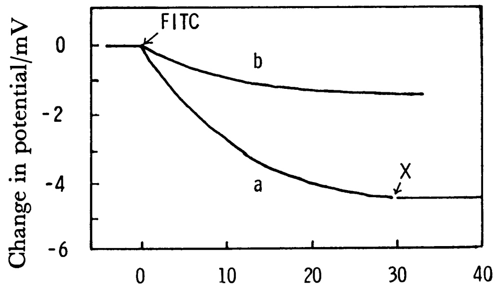


Fig. 2. The change of potential of the aminosilanized titanium electrode caused by the addition of FITC solution. The concentrations of the FITC were 2.1×10^{-4} M for curve a and 7.0×10^{-5} M for curve b. The arrow X indicates the replacement of the solution with a buffer solution.

The vibrating reed electrometer used has the time response less than 0.1 s and the input impedance of $10^5\ \Omega$ or more. The observed change in potential as shown in Fig. 2 is too slow to be explained by the change in the redox level of the solution by the addition of FITC. In addition, the potential induced by the reactant is unchanged by replacement of the solution with a simple buffer solution and remained the same for a long time (Fig. 2a), indicating that the observed potential change is caused by the irreversible reaction on the surface, not by the electrochemical properties of the bulk solution. The impedance yielding the potential for the immuno-electrode was measured to be the order of $10^9\ \Omega$,[5] suggesting an existence of highly insulating layer at the interface between electrode and solution. The potential observed is, therefore, understood by taking into account a surface potential formed by the FITC, and other ions bound on the electrode surface.

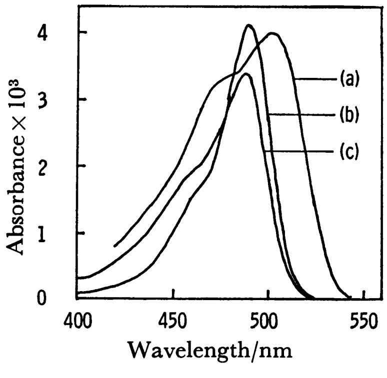
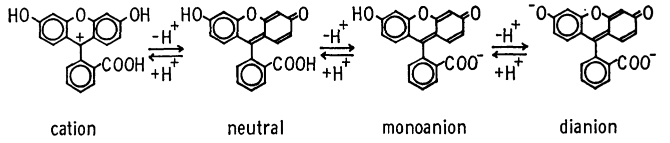


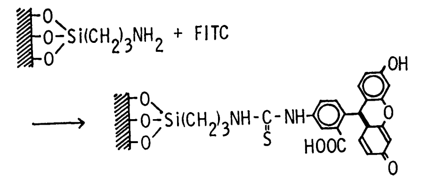
Fig. 3. The absorption spectra of FITC bound on a tin oxide film measured in air (a) and of fluorescein in aqueous solution of pH 5.7 (b) and pH 10.4 (c). The absorption intensities for (b) and (c) are in arbitrary unit.

The absorption spectrum of the aminosilanized tin oxide film that reacted with FITC was studied for a sample immersed in the FITC solution for 90 min followed by washing in the ultrasonic cleaning bath and drying (curve a of Fig. 3). Curves b and c in Fig. 3 show the absorption spectra of fluorescein in aqueous solutions of pH 5.7 and 10.4, respectively. Since the FITC bound on the surface maintains the π-electron network of fluorescein, the absorption spectrum of the bound FITC must be similar to those of fluorescein. Fluorescein in aqueous solutions is in various protolytic equilibria as shown below:



The pH dependence of the absorption and emission spectra of fluorescein in aqueous solution was studied by several investigators.[14–16] Martin and Lindqvist[16] reported that a strong absorption at 491 nm ($\varepsilon=8.8\times10^4$) in the case of an alkaline solution can be identified to the dianion of fluorescein, and that the monoanion existing in a weak acid solution has bands at 475 nm ($\varepsilon=3.1\times10^4$) and at 437 nm. From these assignments the 465 and 500 nm bands of the FITC bound on SnO_2 can be reasonably assigned to the monoanion and the dianion of the fluorescein fragment bound on the solid.

These electrical and spectroscopical results indicate that FITC has certainly reacted with the amino group on the modified solid surfaces as follows.



Assuming that the molar extinction coefficient of the dianion peak of bound FITC is 8.8×10^4, equal to that of fluorescein dianion in alkaline solution, and that of the monoanion of bound FITC is 3.1×10^4 at the absorption peak,[16] the surface coverage of immobilized FITC on the tin oxide and the ratio of the monoanion to dianion can be roughly evaluated from the result in Fig. 3. The resultant coverage is 9.7×10^{-7} mol m^{-2} and the ratio of monoanion to dianion is 1.2: 1, taking account of the fact that the absorbance at 465 nm is expressed by a superposition of the monoanion peak and the dianion tail. Similarly, the coverage on the quartz was estimated to be 6.7×10^{-7} mol m^{-2}, very close to the result on tin oxide. Murray *et al.*[10] estimated from ESCA measurement that organosilanes are bound on the surface to a density of 1 to 2×10^{-6} mol m^{-2} on tin oxide and titanium oxide. Our coverages obtained are in good agreement with their values.

In order to evaluate the reaction rate from absorption spectral measurements, the aminosilanized tin oxide film was immersed into a 4.4×10^{-4} M FITC solution for various periods of time, and the absorption spectrum was measured in air. The optical density at the absorption maximum, 500 nm, of the bound FITC was 1.07×10^{-3}, 4.43×10^{-3}, and 4.71×10^{-3} for the period of 30, 90, and 1440 min, respectively, indicating that the reaction is completed mostly in 90 min, in agreement with the rate of change in electrode potential observed. It was confirmed spectrophotometrically that the FITC thus attached was not removed by washing with water or acetone in the ultrasonic bath for 10 min. In contrast, the tin oxide film which was not modified with organosilane did not show any sign of the absorption spectrum of FITC, indicating that the physical adsorption of FITC onto the tin oxide surface was negligible.

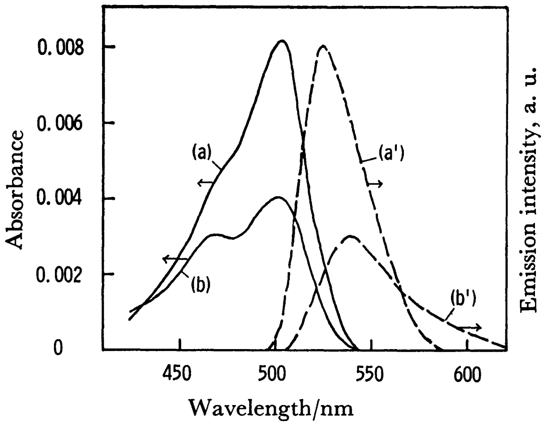


Fig. 4. Absorption and emission spectra of FITC bound on a tin oxide film mesaured in alkaline solution of pH 10.4 : (a) and (a′), and in air : (b) and (b′).

Emission Spectra of the Bound Dye. Figure 4 shows the absorption and emission spectra of FITC bound on tin oxide film measured in air and in a carbonate–hydrogencarbonate buffer solution of pH 10.4. In an alkaline solution of fluorescein, both the absorption peak at 493 nm and the emission peak at 515 nm were assigned to the monomeric dianion by Leonhardt *et al.*[15] and Martin and Lindqvist.[16] The equilibrium constant, pK, between the monoanion and the dianion was reported, from absorption measurement, to be 6.7.[15,16] Therefore, the bound FITC is thought to exist mostly in the dianion form when in contact with the alkaline solution, so that the absorption and the emission bands observed in the alkaline solution (Fig. 4 curve a and a′) are attributed to the dianion. As the emission band measured in air (Fig. 4, b′) is considerably different from that measured in the alkaline solution, it is most reasonably assigned to the monoanion. In the case of titanium oxide powder or quartz used as the substrate, the bound TITC was found to have essentially the same absorption and emission spectra as those in the case of tinoxide film described above.

In the case of FITC bound on a tin oxide film, an emission was observed by excitation with light of 313 nm from a mercury arc lamp. The emission peak was found at 517 nm in water and at 537 nm in air, ascribable to the dye dianion and monoanion, respectively. As the absorption of the dye is very weak at around 313 nm, and the photon energy at 313 nm is much higher than the band gap, 3.2 eV, of tin oxide, the ocurrence of the emission from the bound FITC can only be explained by the excitation of the tin oxide from the valence band to the conduction band followed by the energy transfer from tin oxide to the dye. A similar energy transfer process was pointed out for the system of fluorescein adsorbed on zinc sulfide and zinc oxide crystals by Lendvay.[17]

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