Biosensors and Bioelectronics 26 (2010) 1366–1372

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Direct detection of enzyme-catalyzed products by FET sensor with ferrocene-modified electrode

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ARTICLE INFO

Article history:
Received 13 April 2010
Received in revised form 4 July 2010
Accepted 15 July 2010
Available online 21 July 2010

Keywords:
Extended-gate FET sensor
Enzyme sensor
Redox reaction
Mediator
Alkanethiol
Nernst equation

ABSTRACT

An FET-based biosensor with a ferrocene-modified gold electrode detects the enzyme-produced electrons by using mediators that transfer the electrons from the enzyme to the sensor. Since an extended-gate FET sensor with a light-shielding mask can be operated without a light-shielding box, a small portable instrument will soon be realised. However, when the FET sensor detected enzyme-catalyzed products with the mediators under light conditions, measurements fluctuated due to photo-reduction of the mediators, resulting in decreased sensitivity. To improve sensitivity by reducing the fluctuation, we developed a procedure for directly detecting enzyme-catalyzed products without using the mediators. The key technique used in this procedure was a measurement technique using our developed potential-keeping method, in which the modified electrode of the FET sensor was oxidised by ferricyanide solution to make its surface the same high potential every time, and this high potential was kept until measurement because of the high input impedance of the FET structure. After this method was applied, the interfacial potential of the gold electrode decreased depending on the amount of enzyme-catalyzed products due to the ferrocene molecules immobilised on the gold electrode directly reacting with the products. The results obtained in light conditions indicated that model compounds of the products were detected from 10 μM to 10 mM with the Nernstian response of 59.2 mV/decade. Also, this method was applied to pesticide detection by using the enzyme inhibition by pesticide, and 5 ppb of diazinon was successfully detected by using only a sensor chip.

1. Introduction

Electrochemical enzyme-based biosensors that utilise enzymes as recognition elements have been widely studied. These biosensors are normally based on an enzyme-catalyzed reaction that produces electrons or protons. Ion-sensitive field-effect transistor (ISFET)-based biosensors, which are categorised in enzyme-based biosensors, detect the protons produced by enzyme-catalyzed reactions. The initial concept of the ISFET-based enzyme sensor was proposed by Janata and Moss (1976). Since the first results of using an ISFET-based biosensor for measuring penicillin were reported (Caras and Janata, 1980), it has been developed for detecting various kind of analytes such as glucose, urea, and so on (Dzyadevych et al., 2006). To take advantage of their high sensitivity and ease of integration into analysis systems, the ISFET-based sensors are commonly studied in the field of clinical medicine and in biotechnological and environmental applications (Schöning and Poghossian, 2002). However, since pH change caused by proton production is suppressed by a buffer solution, the sensitivity tends to lower because of its high buffer capacity (Caras and Janata, 1980; Miyahara and Moriizumi, 1985). Even though a solution with high ionic strength and high buffer capacity is suitable for the enzyme activity, it is difficult to operate ISFET-based enzyme sensors in such a solution.

Recently, another type of FET sensor, an FET-based biosensor with a ferrocene-modified gold electrode, has been reported (Ishige et al., 2009). This sensor detects not protons but electrons by using mediators that transfer the produced electrons to the ferrocene molecules on the gold electrode of the FET sensor. Thus, the sensitivity of the FET-based biosensor with a ferrocene-modified gold electrode is hardly affected by pH and buffer capacity. Therefore, the FET-based biosensor with the ferrocene-modified gold electrode can be easily operated while keeping its high sensitivity in suitable buffer conditions for the enzyme activity, which is high ionic strength and high buffer capacity. By applying the FET-based biosensor using ferricyanide and ferrocyanide as mediators to detect enzyme-catalyzed reaction, serum cholesterol was detected with the Nernstian slope of 57 mV/decade, which is 97% of theoretical value.

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doi:10.1016/j.bios.2010.07.053

Meanwhile, since the FET-based biosensor is light sensitive, it must be operated in dark conditions such as in a light-shielding box, which spoils the advantage of its smallness. We also developed an extended-gate FET sensor that can be operated without a light-shielding box by masking the FET structure within a sensor chip (Kamahori et al., 2007). By applying the extended-gate FET sensor to the enzyme-based biosensor, a small portable instrument will soon be realised. However, when the extended-gate FET sensor with the light-shielding mask detected enzyme-catalyzed products with the mediators under light conditions, measurements fluctuated, resulting in a sensitivity decreasing and its applications being more limited. This study found that fluctuation was caused by not only light sensitivity of the FET structure but also photo-reduction of mediators. To improve sensitivity by reducing the fluctuation, we devised a new procedure for directly detecting enzyme-catalyzed products without using mediators. In this procedure, a measurement technique using the potential-keeping method played an important role in the FET-based biosensor with a ferrocene-modified electrode. In this method, the modified electrode of the FET sensor was oxidised by ferricyanide solution to keep its surface at high potential because of high input impedance of the FET structure. Thus, the extended-gate FET sensor with the ferrocene-modified electrode can directly detect the enzyme-catalyzed products without mediators under light conditions. In addition, this procedure was applied to detect pesticide using the enzyme inhibition by pesticide.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals and reagents were used in the experiments: ethanol, potassium chloride, potassium dihydrogenphosphate, disodium hydrogenphosphate, sodium chloride, sodium hydrogencarbonate, sodium hydroxide, potassium hexacyanoferrate (III), potassium hexacyanoferrate (II), diazinon, acetylthiocholine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), eserine sulfate from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); 11-ferrocenyl-1-undecanethiol (11-FUT) from Dojindo (Kumamoto, Japan); and acetylcholine esterase (AChE) from Liofilchem (Abruzzo, Italy).

All reagent solutions were prepared with deionised water (resistively of 18.2 MΩ cm) from an ultra-pure water system (model WD500, Yamato Scientific Co., Ltd., Tokyo, Japan).

2.2. Fabrication of extended-gate FET sensors

Fig. 1 shows a diagram of the extended-gate FET sensor chip (5 mm × 7.5 mm). The extended-gate FET sensor consists of three parts: the gold electrode, the FET structure that transduces the change in the interfacial potential on the gold electrode into a change in the drain current, and the light-shielding mask that protects the FET structure from light irradiation. The sensor chip was fabricated using a semiconductor process. The FET had an n-channel depletion structure (formed by As^+ ion implantation at 120 keV with a dose of 6.0×10^{11} molecules/cm^2) with p-type source and drain areas. The width and length of the channel region (W/L) were 2400 μm and 5 μm, respectively, and the gate insulator of the FET consisted of a 17.5-nm SiO_2 layer, formed by a wet oxidation method, under a 1.3-μm Si_3N_4 layer formed by plasma-enhanced chemical vapor deposition. The light-shielding mask was an 800-nm thick aluminium layer. The gold electrode (400 μm × 400 μm) was fabricated by sputter depositing an adhesion layer of nickel–tungsten alloy and then a gold layer (100 nm, 99.99% purity) on aluminium wire that was connected to the gate

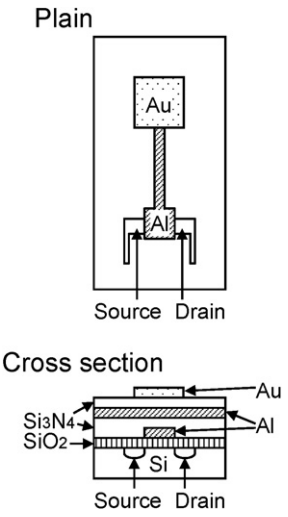

Fig. 1. Diagram of the extended-gate FET sensor chip (5 mm × 7.5 mm), which consists of three parts: a gold electrode, an FET structure, and a light-shielding mask. The FET is connected to the gold electrode (400 μm × 400 μm) by an aluminum wire under a 1.3-μm Si_3N_4 layer.

area of the FET. The fundamental characteristics of an extended-gate FET sensor were described previously (Ishige et al., 2006).

The alkanethiol modification procedure for the gold electrode on extended-gate FET sensor chips was as follows. The sensor chips were kept in an alkanethiol solution for 1 h. The concentration of ferrocenyl-alkanethiol (11-FUT) solution was 5 mM in ethanol. After alkanethiol modification, the sensor chips were rinsed with ethanol and deionised water twice to remove unreacted alkanethiol molecules. The sensor chips were kept in a 0.1-M sodium sulfate solution at room temperature until use.

2.3. Measurement procedure

The FET sensor is light sensitive because of its photo-induced carrier. To investigate light sensitivity of the extended-gate FET sensor with or without a light-shielding mask, gate bias (V_G)–drain current (I_D) curves as characteristics of FET sensors were measured by a semiconductor parameter analyzer (4155C, Agilent, Tokyo, Japan) in 0.1-M sodium sulfate solution with an Ag/AgCl reference electrode containing saturated KCl (RE-1C, BAS, Tokyo, Japan). FET sensors used in measurements were either with or without a light-shielding mask, and the conditions were light (under a fluorescent lamp, FPL36EX-W DK, Hitachi, Tokyo, Japan) or dark (in a light-shielding box). The range of the wavelength of the fluorescent lamp is 400–720 nm.

The interfacial potential of the ferrocene-modified gold electrode on the FET sensor was measured as follows. The drain current of the FET sensor was measured by the semiconductor parameter analyzer with the Ag/AgCl reference electrode to which a 0.1-V DC voltage with a superimposed sine wave (1 MHz, 0.2 V_{p-p}) was applied by a function generator (1940 Multifunction Synthesizer, NF circuit design block, Kanagawa, Japan). A measurement technique using a superimposed high-frequency voltage (Kamahori et al., 2007) was applied to improve the stability of the interfacial potential on the gold electrode of the FET sensor. The measured drain current was converted into the interfacial potential on the basis of the V_G–I_D curve that was measured by the semiconductor parameter analyzer by applying V_G directly to the gold electrode of the extended-gate FET sensor in dry conditions.

To investigate the effect of light on the redox reaction detection system using the FET sensor, interfacial potential of the ferrocene-modified gold electrode on the FET sensor was measured in ferricyanide and ferrocyanide solution used as redox

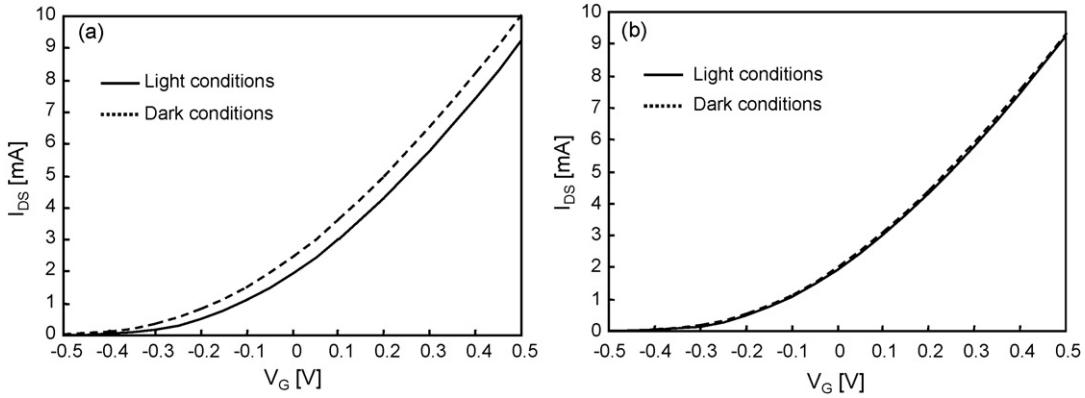

Fig. 2. V_G–I_D curves of FET sensors in dark and light conditions. (a) An extended-gate FET sensor without a light-shielding mask. V_G–I_D curve was shifted to positive potential by light irradiation. (b) An extended-gate FET sensor with a light-shielding mask. V_G–I_D curve did not shift regardless of light irradiation.

solution. The solution contained 0.1-M sodium sulfate as supporting electrolyte and ferricyanide and ferrocyanide as redox compound, the total concentration of which was 10 mM. The concentrations of ferricyanide and ferrocyanide were 0.1 mM and 9.9 mM, 5 mM and 5 mM, and 9.9 mM and 0.1 mM, respectively. Each measurement system was exposed to the light from the fluorescent lamp (FPL36EX-W DK, Hitachi, Tokyo, Japan) for 5 min while the interfacial potential of the ferrocene-modified gold electrode was measured. The solution volume was 2 ml, and aperture size for light irradiation was 1 cm × 2 cm in width and height. The luminance of the light irradiation was 8×10^4 lm/m^2.

Thiol compound as a model compound of an enzyme-catalyzed product was measured by using the potential-keeping method (Fig. 4), which is described in Section 3. First, the ferrocene-modified gold electrode on the FET sensor was oxidised by 10 mM of ferricyanide solution to keep it at a high potential. Next, the sensor chip was dipped in the sample solution for 5 min. Finally, the sensor and Ag/AgCl electrode were set in a measurement solution, and the interfacial potential of the modified electrode was measured in 0.1-M sodium sulfate solution. The sample solutions were PBS containing 6-hydroxy-1-hexanethiol from 10^{-7} M to 10^{-2} M. The same sensor chip was repeatedly used in these measurements.

Diazinon, which is a pesticide, was detected using enzyme inhibition of acetylcholine esterase (AChE) and the potential-keeping method. The sample solution was prepared as follows. First, 10 μl of 5 ppb diazinon as positive control or PBS as negative control and 8 μl of AChE solution was added to 50 μl of PBS. After being stirred, the mixture solution was incubated for 15 min. After the incubation, acetylthiocholine was added as a substrate to the mixture solution, and the mixture solution was incubated for 15 min again. To stop the reaction, 8 μl of 10-mM eserine sulfate solution was added to the mixture solution. The mixture solution was used as a sample solution. The concentration of enzyme-catalyzed products in the sample solution was measured by using the potential-keeping method, and the same sensor chip was repeatedly used. The concentration of enzyme-catalyzed products was also measured using a colour reagent. DTNB solution was added to the mixture solution together with acetylthiocholine. The absorbance of the sample solution was measured, and the concentration of enzyme-catalyzed products was calculated from the absorbance at 450 nm and molar absorbance coefficient (11,725 cm^{-1} mol^{-1}).

3. Results and discussion

3.1. Fluctuation using extended-gate FET-based biosensor in light conditions

FET sensors are usually operated in dark conditions because the FET sensor is light sensitive. When the FET sensor is operated in light conditions, produced photo-carriers in the channel area of FET changes characteristics of FET such as gate voltage (V_G)–drain current (I_D) curve. Since interfacial potential of the sensing electrode of an FET sensor can be obtained from measured characteristics of the FET, the characteristics change causes measurements to fluctuate when FET sensor is used. Fig. 2(a) plots V_G–I_D curves of an FET sensor in dark and light conditions; V_G–I_D curve was shifted to positive potential by light irradiation. The potential shift causes the fluctuation of interfacial potential assumption; therefore, a light-shielding box must be used for precise measurement with an FET sensor. This limitation sometimes results in being unable to realise the small devices.

To overcome this problem, we developed the extended-gate FET sensor with a light-shielding mask (Kamahori et al., 2007) (Fig. 1). Whereas an FET sensor usually has a sensing electrode directly on the FET structure, the extended-gate FET sensor has a gold electrode separated from the FET structure. Therefore, the FET structure of the extended-gate FET sensor can be covered with a light-shielding mask while enabling the gold electrode to still be exposed. Fig. 2(b) shows the characteristics of the extended-gate FET sensor with a light-shielding mask in dark and light conditions. The characteristics did not change regardless of light irradiation. The difference between these characteristics is less than 5 mV.

However, when the extended-gate FET sensor with the light-shielding mask detected enzyme-catalyzed products with the mediators in light conditions, measurements were observed to fluctuate. Fig. 3 shows the interfacial potential of a ferrocene-modified gold electrode on the extended-gate FET sensor with the light-

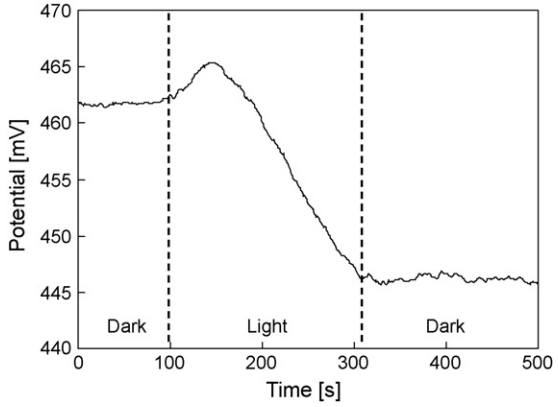

Fig. 3. The interfacial potential of a ferrocene-modified gold electrode on the extended-gate FET sensor with the light-shielding mask in the measurement solution with ferricyanide as a mediator. Whereas the interfacial potential was stable in dark conditions, the interfacial potential decreased in the light conditions.

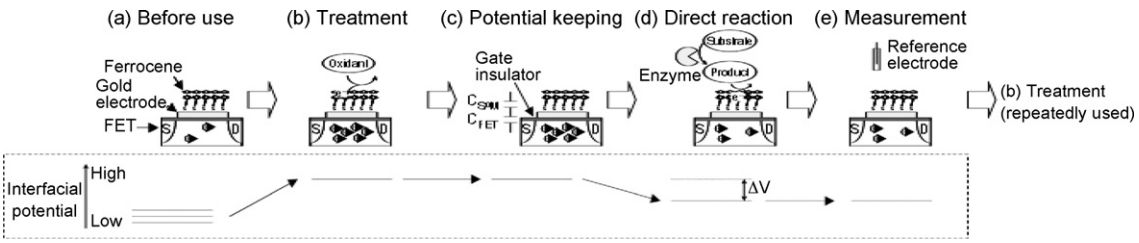

Fig. 4. Proposed measurement procedure for directly detecting enzyme-catalyzed products without mediators using potential-keeping method: (a) The redox state of ferrocene molecules is uncertain, and the interfacial potential of the gold electrode is an unknown value. (b) The ferrocene molecules are oxidised and the interfacial potential becomes a defined high potential. (c) This potential is kept high until measurement due to high input impedance of the FET structure. (d) The ferrocene molecules react with enzyme-catalyzed product, and the interfacial potential of the gold electrode decreases. (e) The interfacial potential is measured using a semiconductor parameter analyzer. Also, the FET sensor chip can be used repeatedly.

shielding mask in the measurement solution with ferricyanide as a mediator. Whereas the interfacial potential was stable in dark conditions, the interfacial potential decreased due to light irradiation in light conditions. After light irradiation finished, surface potential became stable again. The interfacial potential irreversibly decreased about 16 mV in 3 min. Since light sensitivity of an FET sensor is reversible, the observed fluctuation is thought to originate in not the FET sensor but the redox state of mediators. Therefore, we investigated the dependence of the fluctuation in light conditions on the redox state of mediators.

The interfacial potential (E) of the ferrocene-modified gold electrode on the FET sensor depends on the redox state of mediators and follows Eq. (1), which is based on Nernstian response.

$$E = E^{\circ}{}_{\mathrm{med}} + \frac{RT}{F} \ln \frac{[\mathrm{Ox}]_{\mathrm{med}}}{[\mathrm{Red}]_{\mathrm{med}}} \quad (1)$$

where $E^{\circ}{}_{\mathrm{med}}$ is standard electrode potential of mediators, R is gas constant, T is absolute temperature, F is Faraday constant, and $[\mathrm{Ox}]_{\mathrm{med}}$ and $[\mathrm{Red}]_{\mathrm{med}}$ are the concentration of mediators in the oxidised state and reduced state, respectively. When a ferricyanide concentration was much greater than a ferrocyanide concentration (99:1), the interfacial potential decreased in the light conditions at the rate of −0.46 mV/min. Similarly, when a ferricyanide concentration was much smaller than a ferrocyanide concentration (1:99), the interfacial potential decreased at the rate of −1.23 mV/min. On the other hand, when a ferricyanide concentration nearly equaled a ferrocyanide concentration (1:1), the interfacial potential changed as little as −0.08 mV/min. This phenomenon can be explained by photo-reduction of ferricyanide. According to Eq. (1), the interfacial potential is sensitive to ferricyanide or ferrocyanide concentration changing when the ferricyanide concentration is much larger or smaller than the ferrocyanide concentration. On the other hand, the interfacial potential is less sensitive when the ferricyanide concentration is close to the ferrocyanide concentration. The reduction rate of ferricyanide is calculated to be −1.8 μM/min at the ratio of ferricyanide to ferrocyanide as 99:1. On the basis of the experimental conditions, reduction rate of ferricyanide is normalised to be -2.2×10^{-10} mol/(lm min). Therefore, 1 drop (2 mm diameter) of ferricyanide solution under fluorescent light of 1000 lm/m^2 is reduced by light irradiation at 0.7 μM/min. This rate cannot be neglected when detecting a low concentration target.

It was found that the fluctuation of measurement using the extended-gate FET sensor with the light-shielding mask in light conditions is caused by photo-reduction of mediators. Although mediators such as ferricyanide and ferrocyanide are considered to be effective tools for biosensors to widen their application because they mediate various kinds of chemical reactions, high reactivity of mediators often leads to limiting the application of biosensors. The fluctuation of measurement using the FET-based biosensors with mediators, which can be applied to various kinds of enzyme reaction, in light conditions, especially in low concentration targets, is a negative effect of the high reactivity of mediators. To overcome this drawback, we developed a direct detection system for enzyme-catalyzed product that is described in the next section.

3.2. Direct detection of enzyme-catalyzed product with the FET sensor using potential-keeping method

Fig. 4(d) shows the fundamental concept of directly detecting an enzyme-catalyzed product with the FET sensor. The enzyme-catalyzed product directly reacts with ferrocene molecules immobilised on a gold electrode of the FET sensor, and interfacial potential of the gold electrode changes along with the reaction. Therefore, enzyme-catalyzed products can be detected as interfacial potential change without using mediators. However, if the redox state of ferrocene molecules before the reaction is uncertain, the interfacial potential change of the gold electrode becomes indeterminate. To define the redox state of the ferrocene molecules before the reaction, we developed a new measurement procedure using a measurement technique named the potential-keeping method. Fig. 4 shows a schematic image of this measurement procedure. Before use, since the redox state of ferrocene molecules is uncertain, the interfacial potential of the gold electrode is an unknown value (Fig. 4(a)). By treating the FET sensor with an oxidant, the redox state of ferrocene molecules becomes a defined oxidised state, and the interfacial potential of the gold electrode becomes a defined high potential (Fig. 4(b)). This potential is kept high until the ferrocene molecules react with redox molecules in measurement solution because of the high input impedance of the FET structure (Fig. 4(c)). When input impedance of a sensing device is as low as that of a gold electrode without FET structure, interfacial potential decreases due to leakage current. In fact, the interfacial potential became 311 mV with standard deviation of 6.3 mV in 5-times measurement, and the high potential decreased only 7.0 mV/min with standard deviation of 1.3 mV/min. The ferrocene molecules react with the enzyme-catalyzed product as reductant, and the interfacial potential of the gold electrode decreases as the amount of the reduced state of ferrocene molecules on the gold electrode increases due to the redox reaction (Fig. 4(d)) and the interfacial potential is measured using a semiconductor parameter analyzer. Since the redox state of ferrocene molecules before the redox reaction is defined by the potential-keeping method, the amount of enzyme-catalyzed product can be determined with the potential decrease. Also, the FET sensor chip can be used repeatedly because the redox state of ferrocene molecules becomes the same state every time due to the potential-keeping method.

The model compound of enzyme-catalyzed product was measured using the potential-keeping method under light conditions. As shown in Fig. 5, potential change between before and after measurement decreased as the concentration of the model compound

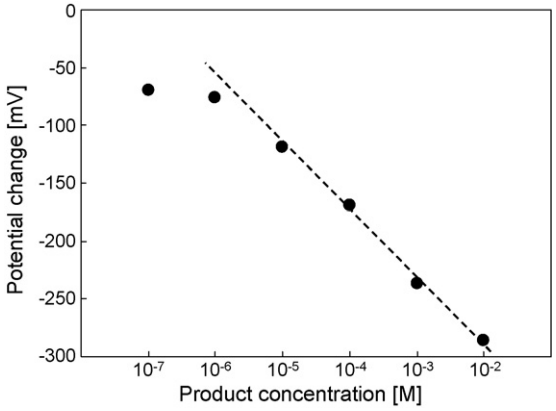

Fig. 5. Potential change versus the concentration of model compound. The potential change decreased as the concentration increased from 10^{-6} M to 10^{-2} M. The slope, plotted as a dashed line, was the same as Nernstian slope of 59.2 mV from 10^{-5} M to 10^{-2} M ($R^2 = 0.9932$).

increased from 10^{-6} M to 10^{-2} M. The measured potential change was linear to the logarithm of the product concentration from 10^{-5} M to 10^{-2} M and the slope, plotted as a dashed line, was the same as the Nernstian slope of 59.2 mV at 25 °C. The limit of detection was about 1 μM, for which sensitivity could not be obtained under light condition using our FET sensors.

Ferrocene molecules are in an equilibrium state between oxidised and reduced state.

$$\mathrm{Fc(II)} \leftrightarrow \mathrm{Fc(III)} + \mathrm{e^-}$$

Therefore, interfacial potential of a ferrocene-modified gold electrode (E) follows Nernst equation.

$$E = E^\circ + \frac{RT}{F} \ln \frac{[\mathrm{Ox}]}{[\mathrm{Red}]} \tag{2}$$

where E° is standard electrode potential, R is gas constant, T is absolute temperature, F is Faraday constant, [Ox] is concentration of ferrocene molecules in oxidised state, and [Red] is concentration of ferrocene molecules in reduced state. After treatment (Fig. 4(b)), the redox state of ferrocene molecules becomes a defined redox state.

$$\frac{[\mathrm{Ox}]^\circ}{[\mathrm{Red}]^\circ} = \text{const.} \tag{3}$$

where $[\mathrm{Ox}]^\circ$ and $[\mathrm{Red}]^\circ$ are concentration of ferrocene molecules in reduced and oxidised state after treatment, respectively. In measurement (Fig. 4(d)), the ferrocene molecules in the oxidised state react with the enzyme-catalyzed product.

$$\mathrm{Fc(III)} + \mathrm{Product(Reduced)} \rightarrow \mathrm{Fc(II)} + \mathrm{Product(Oxidised)}$$

As the reaction rate is proportional to the product concentration, after a constant time, the concentration of ferrocene molecules in each state becomes

$$[\mathrm{Ox}] = [\mathrm{Ox}]^\circ - \alpha[\mathrm{P}] \tag{4}$$

$$[\mathrm{Red}] = [\mathrm{Red}]^\circ + \alpha[\mathrm{P}] \tag{5}$$

where α is a proportionality factor and [P] is the product concentration. Therefore, interfacial potential of a ferrocene-modified gold electrode becomes

$$E = E^\circ + \frac{RT}{F} \ln \frac{[\mathrm{Ox}]^\circ - \alpha[\mathrm{P}]}{[\mathrm{Red}]^\circ + \alpha[\mathrm{P}]} \tag{6}$$

When $[\mathrm{Ox}]^\circ \gg \alpha[\mathrm{P}]$:

$$E \approx E^\circ + \frac{RT}{F} \ln \frac{[\mathrm{Ox}]^\circ}{[\mathrm{Red}]^\circ + \alpha[\mathrm{P}]} = E^\circ + \frac{RT}{F} \ln \frac{[\mathrm{Ox}]^\circ}{[\mathrm{Red}]^\circ} - \frac{RT}{F} \ln \frac{[\mathrm{Red}]^\circ + \alpha[\mathrm{P}]}{[\mathrm{Red}]^\circ} \tag{7}$$

The change of interfacial potential (ΔE) is:

$$\Delta E = -\frac{RT}{F} \ln \frac{[\mathrm{Red}]^\circ + \alpha[\mathrm{P}]}{[\mathrm{Red}]^\circ} = -\frac{RT}{F} \ln \left(1 + \frac{\alpha[\mathrm{P}]}{[\mathrm{Red}]^\circ}\right) \tag{8}$$

When $[\mathrm{P}] \gg [\mathrm{Red}]^\circ/\alpha$,

$$\Delta E = -\frac{RT}{F} \ln \alpha[\mathrm{P}] + \text{const} \tag{9}$$

Therefore, measured potential change was linear to the logarithm of the product concentration from 10^{-5} M to 10^{-2} M, and the slope was the same as the Nernstian slope of 59.2 mV at 25 °C. When $[\mathrm{P}] \approx [\mathrm{Red}]^\circ/\alpha$, the slope becomes smaller. Thus, the equation accurately represents the tendency of the experimental results.

According to the above discussion, the potential-keeping method (Fig. 4(b) and (c)) is very important to directly detect enzyme-catalyzed products without using mediators. In the potential-keeping method, the interfacial potential was set to a defined high potential by the treatment with oxidant, and this high potential was kept until measurement due to high input impedance of the FET sensor. Thus, the amount of enzyme-catalyzed products can be determined as potential change caused by the direct reaction between the products and the ferrocene molecules. Although it has been reported that treatment with 5% hydrogen peroxide solution enables repeated use of FET sensor with a ferrocene-modified gold electrode (Kamahori et al., 2008), the mechanism, especially why the oxidised state of ferrocene molecules is kept until measurement, has not been clear. In this study, it was found that the oxidised state of ferrocene molecules was kept due to high input impedance of the FET sensor. The high input impedance of FET sensor has been utilised to measure the interfacial potential of high impedance sensing silicon nitride or tantalum oxide membrane and ion-selective membrane (Moss et al., 1975). This was the first time the high input impedance of an FET sensor has been utilised to keep a high potential of sensing electrode before measurement. Additionally, the potential-keeping method enabled repeated use of FET sensor.

3.3. Application to pesticide sensor

Various kinds of electrochemical biosensors for pesticide detection are studied and developed (Trojanowicz, 2002). Many of them based on inhibition of enzyme activity and AChE is one of the enzymes whose activity is inhibited by pesticide. For example, when there is no enzyme inhibition, acetylthiocholine is hydrolyzed by AchE, and thiocholine is produced. However, when the activity of AChE is inhibited by pesticides, thiocholine production is reduced or stopped. Thus, pesticide can be detected by measuring thiocholine production. To develop the potentiometric pesticide sensor using our method for direct detection of enzyme-catalyzed products, firstly, we estimated the amount of enzyme-catalyzed product using colour reagent to confirm the amount was in the range of the direct detection method. Then we applied our direct detection method to pesticide detection. We used diazinon (Bruce et al., 1955), which was usually used pesticide for agriculture, as an example of pesticide. Since the residue amount of diazinon in foods is restricted by law (WHO guidelines), it is important to detect the residue in foods using small portable instruments like electrochemical biosensors on site.

The concentrations of thiocholine production were measured using a colour reagent called Ellman's reagent. Fig. 6 shows the

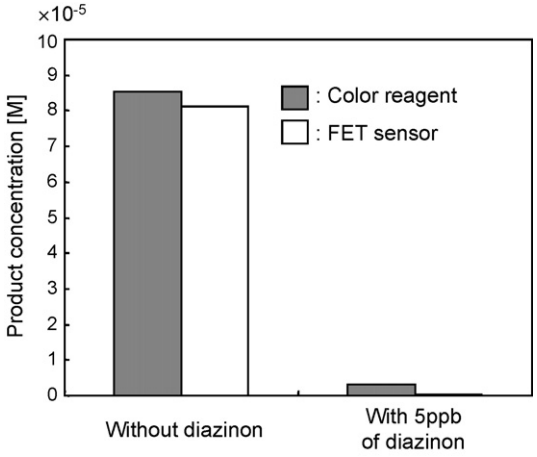

Fig. 6. Concentration of enzyme-catalyzed products with or without pesticide (5 ppb of diazinon). White bar shows the results from the FET sensor, and gray bar shows the results from colour reagent. All data show suppression of enzyme-catalyzed products due to pesticide. By using the FET sensor, potential change without pesticide was −42.5 mV (SD 14.9 mV, $n = 3$) and that with pesticide was −174.3 mV (SD 24.3 mV, $n = 3$). These standard deviations corresponded to 78% and 157% of variation coefficient of concentration, respectively.

results of pesticide detection. Right is without pesticide, and left is with pesticide. Vertical axis shows the concentration. The concentration of 85 μM without pesticide was suppressed to 5 μM by pesticide inhibition. On the basis of the calibration curve in Fig. 5, these concentrations are thought to be distinguishable by the direct detection using the potential-keeping method. Also, concentrations of thiocholine production were directly detected with the potential-keeping method. The potential changes with and without pesticide were −42.5 mV (SD 14.9 mV, $n = 3$) and −174.3 mV (SD 24.3 mV, $n = 3$), respectively. As expected, absolute value of the potential change with pesticide was smaller than that without. Since the difference between with and without pesticide was significantly larger than standard deviation of the potential, pesticide was proven to be detectable by the FET-based pesticide sensor combining the direct detection method with the FET sensor using the potential-keeping method together with enzyme inhibition by pesticide. The concentrations converted from the potential changes with the calibration curve are also shown in Fig. 6 as white bars. The concentrations with and without pesticide were 0 μM and 80 μM, respectively. The results shows colour reagent and potential change agree well. As just described, 5 ppb of diazinon was successfully detected. Since the amount of residue diazinon in foods is restricted below 500 ppb, even considering dilution due to extraction from foods, sensitivity of 5 ppb is enough high for application.

The reproducibility error of pesticide detection was analyzed by estimating the standard deviation of the potential-keeping method. As mentioned in Section 3.2, standard deviation of interfacial potential after the treatment with oxidant was 6.3 mV, and the interfacial potential decreased 7.0 mV/min with standard deviation of 1.3 mV/min. Since an interval time between the treatment with oxidant and the potential measurement was 5 min, the interfacial potential would decreased 35.0 mV with standard deviation of 6.5 mV assuming that decrease rate of the interfacial potential is constant. Therefore standard deviation of the interfacial potential is calculated to be 9.1 mV ($= \sqrt{(6.3\,\mathrm{mV}^2 + 6.5\,\mathrm{mV}^2)}$). It was found that about half of the reproducibility error came from the potential-keeping method and the remaining half would came from the chemical reaction.

Standard deviations of potential changes with and without pesticide corresponded to 78% and 157% of variation coefficient of concentration, respectively. These values are relatively large because potential changes proportional to logarithm of product concentrations. It was found that it was difficult to quantitative measurement of pesticide. However, because the difference of the potential changes between with and without pesticide (131.8 mV) is more than five times of the standard deviation without pesticide (24.3 mV), it was found that pesticide was detected qualitatively.

Most electrochemical biosensors for pesticide detection with enzyme inhibition were based on amperometric detection (Trojanowicz and Hitchman, 1996). Since the signal intensity of amperometric detection is proportional to the electrode area, nanoparticles (Liu and Lin, 2005; Du et al., 2008), boron doped diamond electrodes (Rao et al., 2002), or carbon nanotube electrodes (Du et al., 2007) are used to increase the signal intensity. On the other hand, signal intensity of potentiometric detection does not depend on the electrode area. A potentiometric pesticide sensor with enzyme inhibition measures the enzyme activity through protons, which means pH change, or a mediator (Imato and Ishibashi, 1995; Ristori et al., 1996; Wan et al., 1999). Since pH change is suppressed by buffer solution, a pH potentiometric pesticide sensor cannot be used in high ionic strength solution or high buffer capacity solution. Although a potentiometric pesticide sensor with mediators can be used in high ionic strength solution or high buffer capacity solution (Ivnitskii and Rishpon, 1994), high reactivity of mediators often limits measurement environment as mentioned above. Comparing these types of potentiometric pesticide biosensors, a potentiometric pesticide sensor using the direct detection method can be used with fewer limitations. For example, it can be applied to small pesticide sensors for outdoor use.

4. Conclusion

We directly detected enzyme-catalyzed products without using mediators by using the extended-gate FET sensor with a ferrocene-modified gold electrode. While the current FET sensor responds to light irradiation, the extended-gate FET sensor with a light-shielding mask rarely does. However, the fluctuation of measurement in light conditions due to photo-reduction of mediators remained. Also, we directly detected enzyme-catalyzed products without using mediators by using an extended-gate FET sensor with a ferrocene-modified gold electrode. To specify the relationship between the concentration of enzyme-catalyzed products and the potential change caused by direct reaction between the enzyme-catalyzed products and the ferrocene molecules, the potential-keeping method was developed. In this method, the ferrocene molecules on the FET sensor were oxidised by ferricyanide solution to keep its surface at a defined high potential, and this high potential was kept until measurement because of the high input impedance of the FET structure. Even in light conditions, model compounds of enzyme-catalyzed products can be determined from 1 μM to 10 mM. The slope was the same as a Nernstian response from 10 μM to 10 mM. An FET-based pesticide sensor was developed by combining the direct detection system and enzyme inhibition by pesticide. 5 ppb of diazinon was successfully detected as the potential change of 132 mV, and the results agreed well with those from using colour reagent.

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