〈Research Article〉

# Simple phenol oxidase activity assay method based on a signal accumulation type of ion-sensitive field-effect transistor

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**Summary** A signal accumulation type of ion-sensitive field-effect transistor (SA-ISFET) acts as a sensor capable of detecting changes in protons due to enzymatic reactions. Here, we describe a phenol oxidase activity detection method that uses SA-ISFET and an electron mediator, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). The method enabled simple and cost-effective laccase and bilirubin oxidase assays that could detect activities of 0.005 and 0.05 U/mL, respectively, when potential signals accumulated 10-fold. In addition, assay application to a labeled enzyme detection system based on an immunoassay was discussed.

**Key words:** ISFET, Phenol oxidase, Electron mediator, Laccase, Bilirubin oxidase

## 1. Introduction

The ion-sensitive field-effect transistor (ISFET) is an electronic device that can be used to measure the density of protons in aqueous solutions as altered potentials[1]. ISFET devices are smaller and cheaper than other electronic devices[2]. Recently, a signal accumulation type of ISFET (SA-ISFET, Fig. 1A) with improved sensitivity has been developed, because the sensitivity of ISFET was low (theoretical maximum sensitivity determined according to the Nernst equation was approximately 60 mV/pH unit), and proton density could not be determined with high precision[3-5].

We found that the SA-ISFET sensor could directly convert changes in ion abundance that result from enzymatic reactions into electric signals. Thus, enzymatic reactions could be monitored in real time and only a few microliters of sample were required, which eliminates the need for complicated and troublesome colorimetric detection processes. In previous studies, we developed novel analytical methods based on SA-ISFET technology for measuring creatinine, cholesterol ester, urea, gallic acid, ATPase, RNA polymerase, and horseradish

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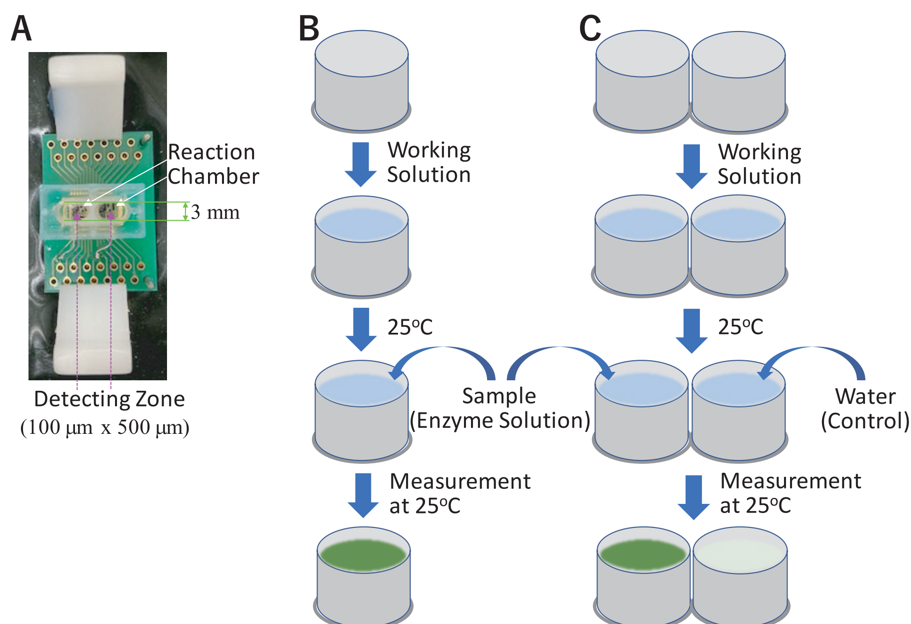



Fig. 1 Sensor and measurement methods. (A) SA-ISFET sensor with two reaction chambers that had 100 μL capacity. (B) The major steps of the one channel method. (C) The major steps of the two channel method.

peroxidase[3-9].

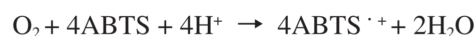
In this paper, we describe an SA-ISFET-based assay for detecting the phenol oxidase activity of laccase and bilirubin oxidase. The assay is simple and cost-effective, and is expected to be applicable to a labeled immunoassay enzyme detection system.

## 2. Materials and Methods

### Materials

Laccase from *Trametes versicolor*, bilirubin oxidase from *Bacillus subtilis*, and tyrosinase from mushrooms were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), Asahi Kasei Pharma (Tokyo, Japan), and Calzyme Laboratories (San Luis Obispo, California, USA), respectively. Laccase from *Trametes* sp. Y120 and bilirubin oxidase from *Trachyderma tsunodae* were provided by Amano Enzyme (Nagoya, Aichi, Japan) and Nipro Corporation (Osaka, Japan), respectively. Two bilirubin oxidases, tyrosinase, and *T. versicolor* laccase were of diagnostic and research grades, whereas *Trametes* sp. Y120 laccase industrial grade and of low purity.

The electron mediator 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Nacalai Tesque, Kyoto, Japan) was used as a phenol oxidase substrate. As shown below, since ABTS consumes four hydrogen ions per molecule in the reaction, it is theoretically possible to detect proton changes by SA-ISFET.



$$O_2 + 4ABTS + 4H^+ \rightarrow 4ABTS^{\cdot +} + 2H_2O$$

The SA-ISFET system used was obtained from Bio-X Inc. (Kyoto, Japan), as reported previously[3-9].

### Methods

In order to maximize the laccase and bilirubin oxidase assay sensitivities, the working solution composition was first optimized. The optimal buffer concentration was determined to be 1 mmol/L, according to previous studies[6-9]. Increasing the buffering capacity above 5 mmol/L greatly reduced the sensitivity. Based on the evaluations of various buffers that were within the pH range of objective enzymes, we chose phthalate (final pH of working solution: 4.0) or monopotassium phosphate (final pH

of working solution: 4.4), which produced a significant potential change (data not shown). The phthalate buffer was easily and inexpensively prepared using a commercially available buffer solution standard (Yokogawa Electric Corporation, Tokyo, Japan). The addition of 50 mmol/L NaCl, as an electrolyte, was also required to maximize assay sensitivity and stability.

A 45 µL volume of working solution composed of 1.5 mmol/L ABTS, 1.0 mmol/L phthalate or monopotassium phosphate, and 50 mmol/L NaCl, was placed into reaction chambers on the SA-ISFET sensor, which had a 100 µL capacity (Fig. 1). The temperature was stabilized at 25°C, and a one or two channel method was selected. For the one channel method, 5 µL of sample was added to the reaction chamber, and potential was measured in real time for 0-300 or 0-240 seconds by accumulation (Fig. 1B). For the two channel method, 5 µL of sample or water (control) was added to each reaction chamber. The potential was measured in real time for 0-300 or 0-240 seconds by accumulating and then subtracting the output potential signals (Fig. 1C). The other settings were as follows: accumulation number, 10; averaging number, 10; and measurement interval, 5 seconds. Statistical processing of the data was performed using Excel.

MOE (Molecular Operating Environment) software ver. 2019.01 (Chemical Computing Group Inc., Montreal, Canada) was used for molecular visualization[10].

## 3. Results

Measurement of laccase activity

Commercially available laccases exhibit high activities at pH 3-5[11]. As shown in the Materials and Methods section, a cheap and easily preparable phthalate buffer was adopted to assay laccase activity. Although significant potential changes were also produced using other buffers, such as monopotassium phosphate and acetate, their reproducibilities were lower than that of phthalate (data not shown).

The time courses of the potential changes with SA-ISFET are presented in Fig. 2A. When *T. versicolor* laccase was used at a final concentration of 0.01 U/mL, the potentials of both the one and two channel methods linearly increased at 26 mV/min. The assay behavior was similar to that of spectrophotometric enzyme assays, although initial potential signals were disturbed due to manual handling. In contrast, 10 U/mL of *Trametes* sp. Y120 laccase was necessary for the potential change of 66 mV/min. This enzyme is of industrial grade for food

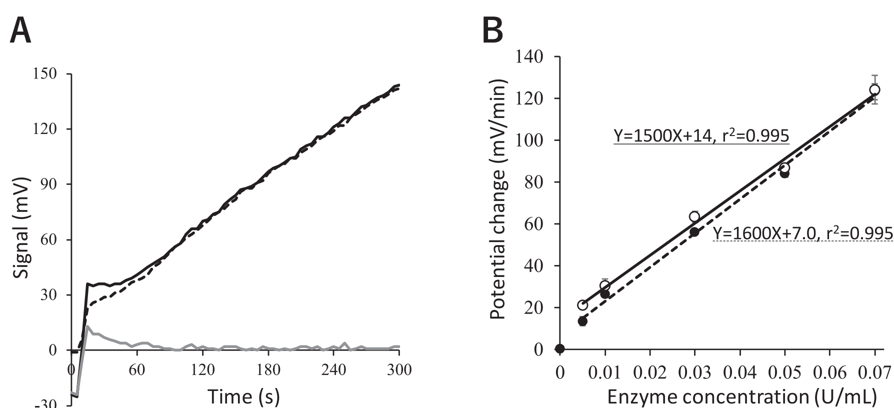



Fig. 2 Time courses and linearities of laccase measurement. (A) Time courses. *T. versicolor* laccase was used as a sample at a final concentration of 0.01 U/mL. Black line; time course of a laccase sample (a result of one channel method), dotted line; time course of two channel method, gray line; time course of a water sample. (B) Linearities. The error bars showed the standard deviation for n = 5 trials. Black line; one channel method, dotted line; two channel method.

processing and chemical synthesis[12]. It is likely that low enzyme purity due to simple process will decrease the potential signal.

The standard curves for the SA-ISFET laccase assay are presented in Fig. 2B. Those of the *T. versicolor* laccase were linear at 0.005-0.07 U/mL, with correlation coefficients of 0.997 (coefficients of determination: 0.995). The inclination of the two channel method was 1.07 times that of the one channel method. In contrast, those of *Trametes* sp. Y120 laccase were linear at 10-40 U/mL, with a correlation coefficient of 0.998 (coefficient of determination: 0.996). The inclination of the two channel method was 1.02 times that of the one channel method. The sensitivity and linearity of the one channel method compared favorably with those of the two channel method.

For the within-day and between-day precision studies, *T. versicolor* laccase samples were assayed 12 times a day and once a day for seven days, respectively, at a final concentration of 0.01 U/mL. The coefficients of variation for the within-day study were calculated to be 9.0 and 7.8% for the one and two channel methods, respectively. On the other hand, those for the between-day study were 13 and 10% for the one and two channel methods. These results indicate that our method is suitable for assaying samples, and the SA-ISFET device can function as an excellent biosensor.

Measurement of bilirubin oxidase and tyrosinase activities

Commercially available bilirubin oxidases exhibit high activities at pH 4-6[13,14]. As shown in the Materials and Methods section, monopotassium phosphate was inexpensive and used to assess bilirubin oxidase activity. The time courses of the potential changes with SA-ISFET are presented in Fig. 3. When *B. subtilis* bilirubin oxidase was used at a final concentration of 0.1 U/mL, the one and two channel method potentials linearly increased at 71 and 79 mV/min, respectively (Fig. 3A). In contrast, when *T. tsunodae* bilirubin oxidase was used at a final concentration of 1.0 U/mL, the one and two channel method potentials linearly increased at 64 and 67 mV/min, respectively (Fig. 3B).

The standard curves for the SA-ISFET bilirubin oxidase assay in the one channel method are also presented in Fig. 3. That of the *B. subtilis* bilirubin oxidase correlated at 0.05-0.2 U/mL, with a correlation coefficient of 0.919 (coefficient of determination: 0.845) (Fig. 3C). In contrast, that of the *T. tsunodae* bilirubin oxidase was linear at 0.4-1.0 U/mL, with a correlation coefficient of 0.972 (coefficient of determination: 0.944) (Fig. 3D). Our results show that the sensitivities, linearities, and reproducibilities of the bilirubin oxidase assays were clearly inferior to those of the *T. versicolor* laccase assay.

Activity measurement of tyrosinase, which is a phenol oxidase, was also attempted using our method. However, no potential change was detected (data not shown).

Application of the laccase assay method

The laccase and bilirubin oxidase assay methods that were developed in this study are expected to be applicable to immunoassay labeled enzyme detection systems. On the other hand, laccase is useful in bioremediation applications, including the biodegradation and detoxification of environmental pollutants[15,16]. Its application requires inexpensive natural compounds with low environmental impact, rather than conventional chemical mediators.

As shown in Fig. 4, the SA-ISFET-based laccase assay detected no potential signal without mediator and exhibited a high response signal with ABTS. It was demonstrated to be compatible as a screening system of natural mediators[15,16].

## 4. Discussion

In this study, a new method that used SA-ISFET for assaying phenol oxidase activity was devised and successfully detected laccase and bilirubin oxidase activities. This method will be useful as an effective sensor of enzyme activity, because it makes use of simple and inexpensive reagents, as compared to other conventional methods. Moreover, the SA-ISFET sensor system provides an apparatus

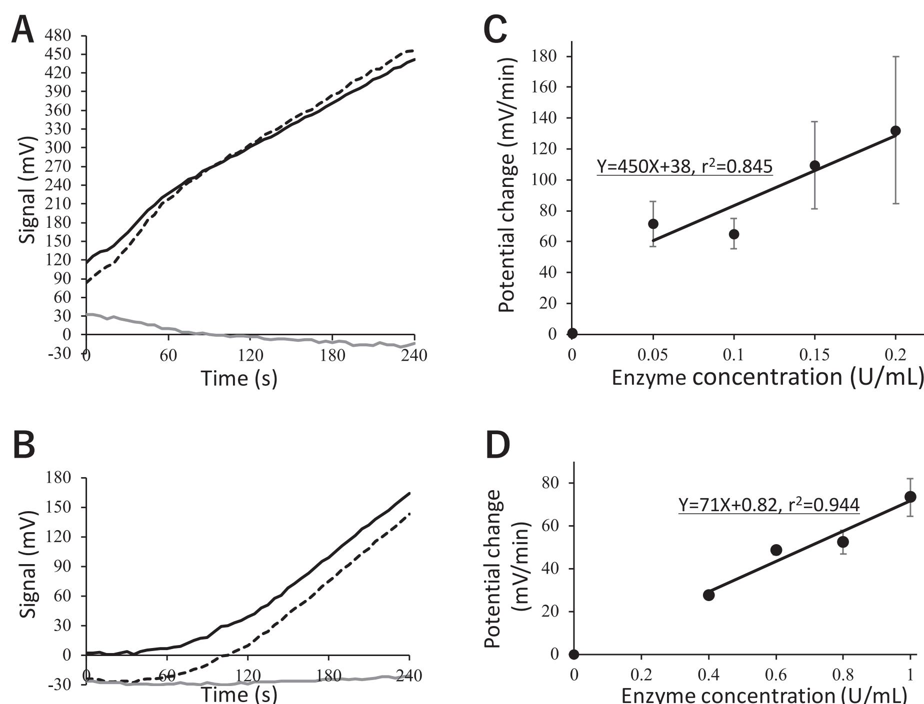



Fig. 3 Time courses and linearities of bilirubin oxidase measurement. (A) Time courses of *B. subtilis* bilirubin oxidase, which was used as a sample at a final concentration of 0.1 U/mL. Black line; time course of an enzyme sample (a result of one channel method), dotted line; time course of two channel method, gray line; time course of a water sample. (B) Time courses of *T. tsunodae* bilirubin oxidase, which was used as a sample at a final concentration of 1.0 U/mL. Black line; time course of an enzyme sample (a result of one channel method), dotted line; time course of two channel method, gray line; time course of a water sample. (C) Linearity of *B. subtilis* bilirubin oxidase in the one channel method. The error bars showed the standard deviation for n = 5 trials. (D) Linearity of *T. tsunodae* bilirubin oxidase in the one channel method. The error bars showed the standard deviation for n = 5 trials.

smaller than conventional optical analyzers.

Peroxidase is used as an enzyme for immunoassays in the fields of basic biochemistry and clinical testing. We previously developed the SA-ISFET sensor system for assaying peroxidase activity by selecting ABTS as an electron mediator that could be applied in the immunoassay enzyme activity detection[9]. Furthermore, a SA-ISFET-based device was constructed to efficiently detect increases and decreases in proton concentration before and after enzymatic reaction and thereby evaluate various reactions. Hydrogen peroxide is an unstable reagent that is included with ABTS, and hence, the previous peroxidase assay method must adopt a two-reagent system for long-term storage. The new method does not require hydrogen peroxide, unlike the previous method.

Table 1 Relative sensitivities of enzyme assay methods

| Enzyme | Origin | Relative sensitivity |
|---|---|---|
| Peroxidase | Horseradish | 0.24 |
| Laccase | *T. versicolor* | 1.0 |
| Laccase | *Trametes* sp. Y120 | 0.049 |
| Biliruvin oxidase | *B. subtilis* | 0.42 |
| Biliruvin oxidase | *T. tsunodae* | 0.24 |

Each sensitivity was estimated by calculating potential change (mV/min) per enzyme protein concentration (mg/mL).

The sensitivities of the five enzyme assay methods with SA-ISFET (horseradish peroxidase and four types of laccases and bilirubin oxidases) were compared (Table 1). As a result, *T. versicolor*

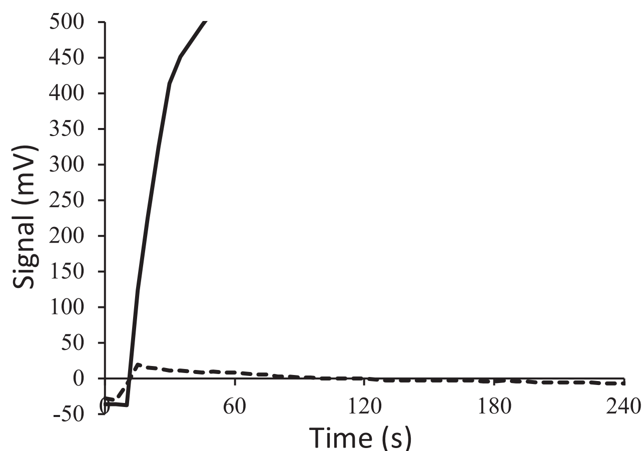



Fig. 4 Comparison of laccase measurements with and without a mediator. A 45 μL volume of working solution composed of 1.1 U/mL *T. versicolor* laccase, 1.0 mmol/L phthalate, and 50 mmol/L NaCl was placed into the reaction chamber on the SA-ISFET sensor. A 5 μL volume of 14 mmol/L ABTS or water was added as a sample to the reaction chamber, and the potential was measured in real time for four minutes by accumulation (solid or dotted line).

laccase activity was measured with highest sensitivity. Measurement of *B. subtilis* bilirubin oxidase also exhibited higher sensitivity than that of horseradish peroxidase. In order to perform immunoassays, an antibody-enzyme conjugate must be constructed. If laccase and bilirubin oxidase antibody labeling techniques are established for immunoassays, the SA-ISFET detection systems of these enzymes would be preferred.

Preparation of reduced IgG and enzyme maleimide activation using a cross-linking reagent for maleimide and active ester groups, such as N-(6-maleimidocaproyloxy)succinimide, are necessary to construct enzyme-conjugated IgGs. The X-ray crystal structure of *T. versicolor* laccase has been determined with high resolution[17]. To identify targets for modification, we confirmed lysine residues, with which active esters will form covalent bonds (Fig. 5). Cross-linking reagents could bind to lysine residues near the active center, thereby blocking substrate access to the active site. All eight lysine residues of *T. versicolor* laccase were located on the surface of the protein and were not proximal to the

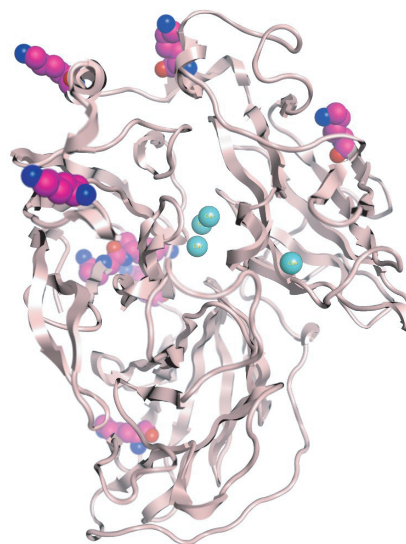


Fig. 5 Overall tertiary structure of laccase. The X-ray crystal structure of *T. versicolor* laccase (PDB ID: 1GYC) is represented by a ribbon model. Four cofactor copper ions and eight lysine residues are shown by cyan balls and space-filling models, respectively.

active center, especially the four copper ion cofactors. Thus, enzyme activity losses are not expected after maleimide activation.

## Conflicts of interest

The authors have no conflicts of interest.

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