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Sensitive D-Amino Acid Biosensor Based on Oxidase/Peroxidase

System Mediated by Pentacyanoferrate-bound Polymer

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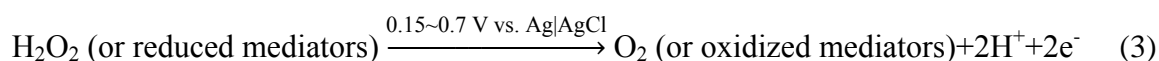E-mail Address: kano.kenji.5z@kyoto-u.ac.jp

Abstract

A sensitive D-amino acid oxidase (DAAO)/peroxidase (POD) bienzyme biosensor is constructed, in which pentacyanoferrate-bound poly(1-vinylimidazole) polymer ($PVI[Fe(CN)_5]$) is selected as a mediator. Reductive current of $PVI[Fe(CN)_5]$ related to the H_2O_2 concentration generated in the DAAO reaction was measured at -0.1 V vs. Ag|AgCl with $DAAO/POD/PVI[Fe(CN)_5]$-modified electrode. The result revealed that $PVI[Fe(CN)_5]$ is suitable as a mediator for this bienzyme system due to its appropriate formal potential and its extremely low reactivity against DAAO. The stability of DAAO was improved by adding free flavin adenine dinucleotide and the electrode composition was optimized for the detection of D-alanine. Nafion and ascorbate oxidase-immobilized films worked successfully to prevent severe interference from uric acid and ascorbic acid. The low detection limits of D-alanine (2 μM) and D-serine (2 μM) imply its possibility for the determination of extremely low concentration of D-amino acids in physiological fluids. The proposed bienzyme biosensor is proved to be capable of detecting D-amino acids in urine.

1.   Introduction

The role of D-amino acids (DAAs) has become of great importance in life science, since several DAAs are found to have physiological functions in mammals. With regard to the central nervous system in brain, D-serine acts as a co-agonist of *N*-methyl-D-asparate receptor, which is associated with learning and memory (Bains and Oliet, 2007), and D-alanine in the anterior pituitary gland, pancreas, and plasma might have a physiological function to the insulin regulation (Hamase et al., 2009). The concentration of DAAs also shows a strong correlation to some diseases. For example, the ratio of D-serine to the total (D-+ L-) serine concentration in the serum of Alzheimer's disease patients was reported to be lower than that of normal subjects (Hashimoto et al., 2004). As the dynamic monitoring of the DAAs concentration is of acute interest, a rapid, simple and highly sensitive method for the DAA detection is essential for further study. Separation-based analytical methods such as high performance liquid chromatography (HPLC) and capillary electrophoresis are conventionally used for DAA determination with high sensitivity (Hashimoto et al., 1992; Quan et al., 2005). D-Amino acid oxidase (DAAO) oxidizes DAAs to the corresponding imino acids, which have been further utilized to react with *o*-phenylenediamine and 2-mercaptoethanol to form the corresponding fluorescent quinoxalinol derivatives. The fluorescent derivatives can be

separated by HPLC and this method eliminates the interference from extremely high concentrations of L-amino acids (Kato et al., 2010; Oguri et al., 2005). Considering the convenience for *in vivo* detections and the undesirability of cumbersome instruments, enzyme-based amperometric biosensors are favored for DAA detection. DAAO is a flavoenzyme and is widely used in enzymatic biosensors for DAA determination, either based on the direct detection of generated H_2O_2 (Pernot et al., 2008; Sacchi et al., 1998; Zain et al., 2010) or the detection of artificial mediators used as electron acceptors of the substrate-reduced DAAO (Eqs. 1-3) (Arai et al., 1998; Murthy and Sharma, 1999).

$$DAAs + DAAO(FAD) \rightarrow imino\ acids + DAAO(FADH_2) \tag{1}$$

$$DAAO(FADH_2) + O_2\ (or\ mediators) \rightarrow DAAO(FAD) + H_2O_2\ (or\ reduced\ mediators) \tag{2}$$

$$H_2O_2\ (or\ reduced\ mediators) \xrightarrow{0.15\sim0.7\ V\ vs.\ Ag|AgCl} O_2\ (or\ oxidized\ mediators) + 2H^+ + 2e^- \tag{3}$$

However, the oxidation of both H_2O_2 and mediators requires high operation potential, which always accompanies the co-oxidation of other electroactive metabolites in physiological fluids. In addition, the exclusion of O_2 in the case of mediated biosensors seems to be impractical for *in vivo* analysis.

On the other hand, peroxidase (POD) in combination with H_2O_2–producing oxidases including DAAO has been studied intensively in recent years for the substrate detection in the way of direct or mediated electron transfer (Dominguez et al., 2001;

Kacaniklic et al., 1994). As shown in Eq. 4, in mediated bienzyme biosensors, H_2O_2 is reduced by POD and the oxidized mediators with a suitable formal potential can be reduced on an electrode at low operating potentials around 0 V vs. Ag|AgCl.

$$H_2O_2+\text{mediators} \xrightarrow{\text{POD}} H_2O+\text{oxidized mediators} \tag{4}$$

Since the low potential operation decreases the background current and noise levels, and eliminates the undesirable oxidation of electroactive interferents, bienzyme biosensors show high sensitivity and stability. However, such mediated bienzyme sensors may have one problem to be overcome; the oxidized mediators (in Eq. 3) may also act as electron acceptors of H_2O_2-producing oxidases based on the dehydrogenase activity of the oxidase (Matsumoto et al., 2002; Ohara et al., 1993b). The reaction diminishes the cathodic current to interfere the determination of the substrate concentration. In consideration of 1) the possibility of cross reaction of mediators with POD and oxidases, 2) the interference effect of O_2 reduction around -0.2 V (vs. Ag|AgCl), and 3) the occurrence of oxidation of interferents at positive potentials, it becomes an important issue for mediated bienzyme biosensors to select an appropriate mediator with high selectivity for POD alone and a suitable operating potential in the narrow range from -0.2 to 0 V.

Here, we focus our attention on pentacyanoferrate-bound poly(1-vinylimidazole)

polymer (PVI[Fe(CN)$_5$]), which has been synthesized in our group (Ishibashi et al., 2008), as an mediator for the DAAO/POD bienzyme biosensor considering its suitable formal potential and the poor mediating capability of ferricyanide for other flavoenzymes such as glucose oxidase (Kulys and Cenas, 1983; Nieh et al., 2012). PVI[Fe(CN)$_5$] is one kind of electro-conducting hydrogels which can covalently bind enzymes through poly(ethylene glycol) diglycidyl ether (PEGDGE) and shuttle electrons between enzymes and electrode (Ohara et al., 1993a). In this work, the interactions of PVI[Fe(CN)$_5$] with POD and DAAO will be studied, and the electrode optimization and the interference effect will also be investigated.

2. Experimental

2.1 Reagents

2,2'-Azobisisobutyronitrile (AIBN), sodium pentacyanonitrosylferrate(III) dihydrate ($Na_2[Fe(CN)_5(NO)]\cdot 2H_2O$), D-serine, ascorbic acid (AA), uric acid (UA), glutaraldehyde (GA, 20%), and flavin adenine dinucleotide (FAD) disodium salt were obtained from Wako Chem. Co. (Osaka, Japan). 1-Vinylimidazole, DAAO from porcine kidney (8.2 U mg^{-1}), PEGDGE, D-alanine, and Nafion (5 wt% in mixture of lower aliphatic alcohols and water, contains 45% water) were from Sigma-Aldrich (USA). Peroxidase from horseradish (POD, 257 U mg^{-1}) and ascorbate oxidase from *cucumis* sp. (AO, 333 U mg^{-1}) were purchased from Toyobo Co. (Osaka, Japan). UA solution was prepared with 10 mM NaOH, FAD was dissolved in distilled water, and the enzymes, substrates, AA and PEGDGE solutions were prepared with 30 mM potassium phosphate buffer (pH 7.0). Other chemicals were of analytical grade and used as received. The urine sample was donated from a healthy volunteer.

2.2 Synthesis of PVI[Fe(CN)$_5$]

Poly(1-vinylimidazole) (PVI) was synthesized as reported (Ohara et al., 1993a). In brief, a mixture of 1-vinylimidazole and 0.5 g of AIBN (6 mL) was heated under stirring at 70 °C for 2 h in Ar. The yellow precipitate was then dissolved in methanol, followed

by adding dropwise to acetone under strong stirring to get white soft precipitate. PVI powder was obtained after filtering and drying.

PVI[Fe(CN)$_5$] was then synthesized according to the literature (Ishibashi et al., 2008). Briefly, 100 mg of Na$_2$[Fe(CN)$_5$(NO)]·2H$_2$O and 94 mg of PVI were dissolved in 25 mL of 0.6 M NaOH and refluxed at 65 °C for 24 h. The solution was then dialyzed against distilled water for 24 h to remove unreacted compounds. After filtration to remove red precipitate, the suspension was vacuum freeze-dried at −40 °C for 24 h to get PVI[Fe(CN)$_5$] powder. The ratio of the imidazole unit in PVI to the Fe(CN)$_5$ complex was 4.3 as measured by elemental analysis. The stock solution of PVI[Fe(CN)$_5$] was prepared in distilled water.

2.3 Coimmobilization of POD, DAAO and PVI[Fe(CN)$_5$]

A solution containing PVI[Fe(CN)$_5$], PEGDGE, POD and DAAO was cast onto the surface of a glassy carbon electrode (3 mm diameter, BAS) and well mixed with a syringe needle. The electrode was dried at 4 °C for 24 h. Before measurements, the proposed electrode was immersed into a 30 mM potassium phosphate buffer (pH 7.0) for 20 min.

2.4 Fabrication of interference-free bienzyme electrode

To improve the stability of DAAO, 1 μL of 400 μM FAD solution (4×10^{-10} mol, ca. 0.3 μg) was cast onto the proposed electrode fabricated in section 2.3 (see section 3.2 for details). For interference tests, 3 μL of Nafion solution diluted by distilled water was then added onto the electrode after air-drying. Unbounded polymer and enzymes were removed by dipping the electrode into distilled water and 3μL of diluted Nafion solution was cast onto the electrode again after air-drying. Finally, AO was crosslinked with GA on the electrode at 4 °C for 2 h.

2.5 Electrochemical measurements

Electrochemical experiments were carried out in a potassium phosphate buffer (pH 8.0, 30 mM) under stirring at 37 °C with an electrochemical analyzer (611B or 1000, CH Instrument, USA). The reference and counter electrodes were an Ag|AgCl|sat. KCl and a Pt wire, respectively. Cyclic voltammetry was performed at a scan rate of 20 mV s^{-1}.

Oxygen depletion due to the DAAO reaction was measured at 37 °C and −0.6 V with a Clark-type oxygen electrode (Optoscience, Japan). The pH 8.0, 30 mM potassium phosphate buffer solution containing 3 μg mL^{-1} DAAO and 4 mM D-alanine was used for experiments.

3.   Results and discussion

3.1 Reactivities of PVI[Fe(CN)$_5$] against DAAO and POD

Such oxidase/POD bienzyme systems may suffer from interference due to the dehydrogenase activity of oxidase. As shown in Scheme 1(hollow arrows), the oxidized form of some mediators may accept electrons from DAAO, which interferes the substrate detection. In this sensing system, PVI[Fe(CN)$_5$] was selected as a mediator. Figure 1A shows that PVI[Fe(CN)$_5$] has extremely low reactivity against DAAO, while in the POD reaction the catalytic current is clearly observed with PVI[Fe(CN)$_5$] (Fig. 1B). The result indicates the poor interaction between DAAO and PVI[Fe(CN)$_5$]. The quite different reactivities of PVI[Fe(CN)$_5$] against DAAO and POD are interpreted as follows: The redox center (FAD) of DAAO locates in hydrophobic surroundings and is deeply buried in the interior to induce the steric hindrance (Mizutani et al., 1996); PVI[Fe(CN)$_5$] with negatively charged ligands would be difficult to enter into the FAD-catalytic center. Therefore, no obvious mediating effect of PVI[Fe(CN)$_5$] was observed for the DAAO reaction. On the other hand, the redox center (protoheme group) of POD locates near the enzyme exterior with widely open entrance; furthermore, the electrostatic potential around the redox center is positively charged (Carlsson et al., 2005), which makes it easier for PVI[Fe(CN)$_5$] to shuttle electrons between POD and

10

electrode. Thus, we can conclude that PVI[Fe(CN)$_5$] is very suitable as the mediator for the DAAO/POD bienzyme biosensor with high specificity.

### 3.2 Stability of DAAO/POD/ PVI[Fe(CN)$_5$]-modified electrode

DAAO/POD/PVI[Fe(CN)$_5$]-modified electrode has been successfully constructed herein for the DAA detection. However, as shown in Fig. 2A, the current response of D-alanine decreased with successive operations (open square); the current value in the third detection is only two third of that in the first detection. We considered that the poor repeatability of the detection is mainly due to the non-covalent binding property of FAD with DAAO (Arai et al., 1998). Since the binding between FAD and apo-DAAO is weak, FAD gradually releases from holo-DAAO to the bulk solution to lead the decrease in the DAAO activity. Considering keeping the activity of DAAO, sufficient amount of FAD was added into the experimental solutions to avoid the leakage of FAD from holo-DAAO. The effect of the FAD addition was checked by detecting the oxygen depletion in the DAAO reaction measured with the Clark-type oxygen electrode (Fig. 2B). At a low concentration of DAAO without FAD addition (dash line), the activity of DAAO gradually decreased since FAD easily released from DAAO to the bulk solution. On the other hand, the oxygen depletion rate of the DAAO reaction increased and DAAO became more stable in the presence of 40 μM FAD (solid line). The high oxygen

depletion rate in the FAD-containing solution indicates that the addition of FAD keeps the DAAO activity. Good repeatability of the proposed biosensing system was then proven by addition of FAD in the test solution, as evidenced in Fig. 2A (closed rhombus); no drastic decrease in the current response was observed in the successive experiments. Therefore, the experimental solution containing 40 μM FAD was selected in the following experiments for other optimization.

Considering the fact that the biosensor sensitivity is related to the deposited polymer thickness, the composition of POD and DAAO, and the total enzyme capacity, the dependence of the current response on the amount of $PVI[Fe(CN)_5]$, the ratio of POD to DAAO, and the weight ratio of the enzymes to the total weight were examined. The results are shown in Fig. S1. The optimized values were determined as follows: 20 μg of $PVI[Fe(CN)_5]$, 0.1 (the ratio of POD to DAAO), and 0.6 (the weight ratio of the enzymes to the total weight). The final composition of proposed electrode contains 20 μg of $PVI[Fe(CN)_5]$, 10 μg of PEGDGE ,1.07 U of POD and 0.33 U of DAAO.

3.3 Interference effect

The DAA bienzyme biosensor at low operating potential minimizes the undesirable oxidation of electroactive reducing interferents in physiological samples. However, some interferents may react as electron donors for POD to affect the detection.

$$\text{interferents} + H_2O_2 \xrightarrow{\text{POD}} \text{oxidized interferents} + 2H_2O \qquad (5)$$
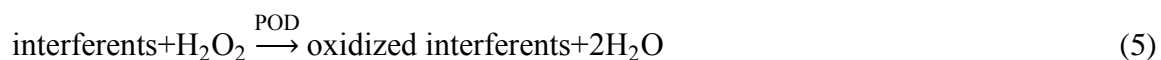
This reaction interferes with the electron transfer between reduced PVI[Fe(CN)$_5$] and POD and causes the underestimation of the DAA concentration. To eliminate the interference effect, negatively charged Nafion was used as a protecting film on the top of the bienzyme electrode to exclude anionic interferents such as AA and UA based on electrostatic repulsion. In addition, 0.3 μg of FAD was cast between the bienzyme layer and the Nafion film in place of the FAD addition in the experimental bulk solution. Furthermore, regarding the low oxidation potential of AA, 15 μg of AO was immobilized with GA on the top of the Nafion film to minimize the interference effect of AA. In separated experiments, it has been proved that PVI[Fe(CN)$_5$] works neither as a substrate (reduced form) for AO nor as an electron acceptor of AO in the catalytic oxidation of AA (data not shown). Since PVI[Fe(CN)$_5$] shows low reactivity against AO, AO utilization does not affect the DAA detection. The interference effect on the amperometric response with different concentrations of Nafion is shown in Table 1; the current response of 10 μM D-alanine decreased with an increase of the Nafion amount because of the thick film which decreases the mass transfer of the substrate. Comparing the response of D-alanine with that in the AA-containing solution, the relative response increased with an increase of the Nafion amount; thick Nafion film shows a better

13

protecting effect. The relative responses over than 100% with very thick Nafion films may be due to the partial desorption of the Nafion film in succeeding measurements for the DAA+AA mixture, which increases the penetration of D-alanine. Taking the current response and the protecting effect into account, 0.8% Nafion was selected for the biosensor fabrication, and no significant interference was observed in the 10 μM D-alanine solution containing 500 μM UA and 100 μM AA (data not shown). The physiologically normal concentration of UA in urine is about 30 times higher than DAAs, and the normal concentration of AA is in the same level with DAAs (Bruckner and Schieber, 2001; Westerman et al., 2000; Zuo et al., 2011). Therefore, this bienzyme biosensor protected by Nafion and AO seems to satisfy the DAA determination in urine.

3.4 Sensors for DAAs

The proposed bienzyme biosensor protected by Nafion and AO has successfully worked for the DAA determination in the presence of high concentrations of interferents. However, in the preliminary test of urine sample, the sensitivity decreased gradually with successive measurements (data not shown). Some molecules in the urine sample may nonspecifically adsorb on the AO layer of the electrode, which fouled the electrode to affect the sensitivity. To eliminate the nonspecific adsorption, 6 μL of 0.8% Nafion was cast on the top of the AO layer for protection. The illustrated structure of the DAA

biosensor is shown in Scheme 2. Most of the interferents are inhibited from entering into the sensing part by the outer Nafion film, and the residual UA and AA are blocked by the inner Nafion film and oxidized by AO, respectively. Thus, DAAs can successfully be detected without interference effect.

Amperometric responses of two major DAAs in human body, D-serine and D-alanine, are presented in Fig. S2, in which the current was measured with the optimized biosensing electrode at −0.1V. The detection limits of D-serine and D-alanine are both 2 μM (S/N > 3); the linear ranges are from 10 to 350 μM and 5 to 150 μM, and the slopes are 5.4 and 9.7 nA μM^{-1}, respectively. It shows that the biosensor has very low detection limit and high sensitivity, which is suitable for the detection of extremely low concentrations of DAAs in physiological samples. The different reactivities of different DAAs indicate that it is difficult for DAAO-based biosensors to determine the total concentration of DAA. Nevertheless, it is still benefit to easily determine the total performance of DAAs with selecting one of DAAs, such as D-alanine, as a standard.

The standard-addition method was then used for a urine sample test. D-Alanine was successively injected into the urine solution which was 100 times diluted with 30 mM potassium phosphate buffer (pH 8.0) and the slope is similar to that measured in the standard D-alanine solution (Fig. S3). The concentration of DAAs in the urine was

evaluated to be 147±16 μM, when D-alanine was selected as a standard. The sensitivity of biosensor is not affected by the interferents in urine. The DAAO/POD bienzyme biosensor is applicable for clinical diagnosis.

4. Conclusion

DAAO/POD bienzyme biosensor has successfully been constructed for the DAA determination. PVI[Fe(CN)$_5$] is suitable as a mediator and for enzyme immobilization based on its appropriate formal potential and poor reactivity with DAAO. The interference effect has been eliminated by the Nafion films and AO, and the biosensor has low detection limit and high sensitivity, which indicates its applicability for clinical diagnosis. Since the main species of DAAs in human brain is D-serine, the proposed biosensor seems to have a potential for D-serine determination in brain without the problem of the DAA specificity of DAAO.

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Figure captions:

Scheme 1

Possible reactions in mediated DAAO/POD bienzyme sensor.

Figure 1

Cyclic voltammograms of (A) DAAO/PVI[Fe(CN)$_5$]-modified electrode in an Ar-saturated solution and (B) DAAO/POD/PVI[Fe(CN)$_5$]-modified electrode in an Air-saturated solution. The dash line is the detection in a buffer solution and the solid line is the detection in 4 mM D-alanine (pH 8.0). Electrode composition, DAAO: 0.33 U, POD: 2.14 U, PEGDGE: 10 μg, PVI[Fe(CN)$_5$]: 20 μg. Scan rate is 20 mV s^{-1}.

Figure 2

(A) Dependence of the current response at –0.1 V on the successive measurements in the solution containing 40 μM FAD and 5 μM D-alanine (closed rhombus) or 5 μM D-alanine only (open square). Electrode composition, DAAO: 0.25 U, POD: 2.67 U, PEGDGE: 10 μg, PVI[Fe(CN)$_5$]: 30 μg. (B) Time dependence of the dissolved oxygen concentration during the DAAO reaction in the solution containing 40 μM FAD and 4 mM D-alanine (solid line) or 4 mM D-alanine only (dash line).

Scheme 2

The composition and the reaction sequence of the proposed DAAO/POD bienzyme
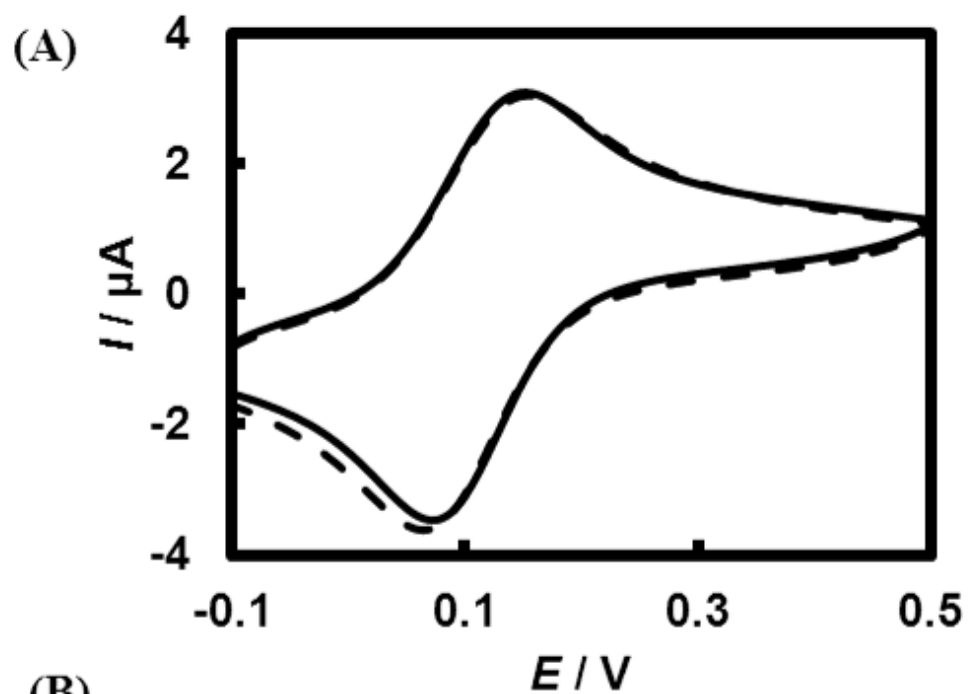
biosensor protected by Nafion and AO.

Scheme 1

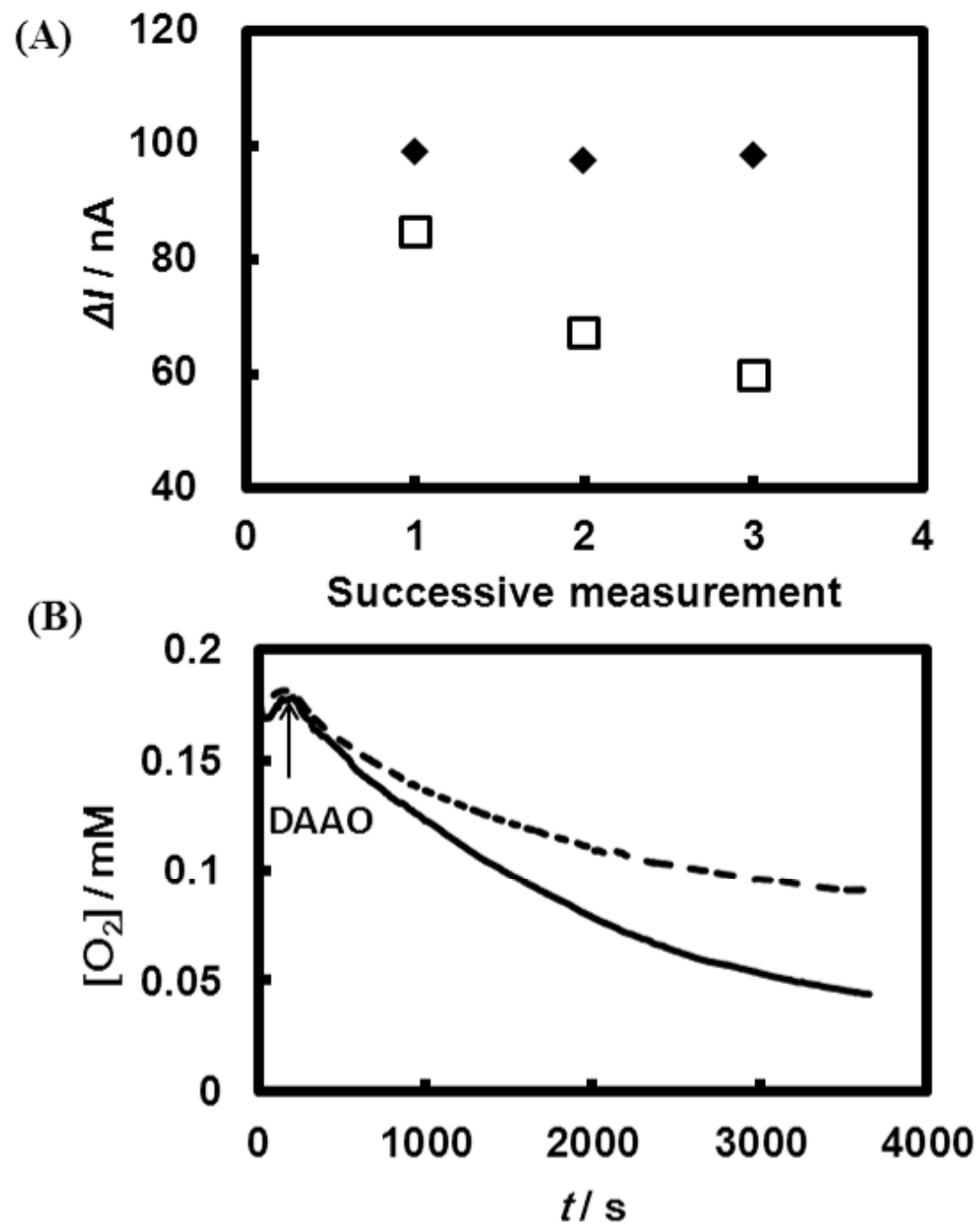**(A)**
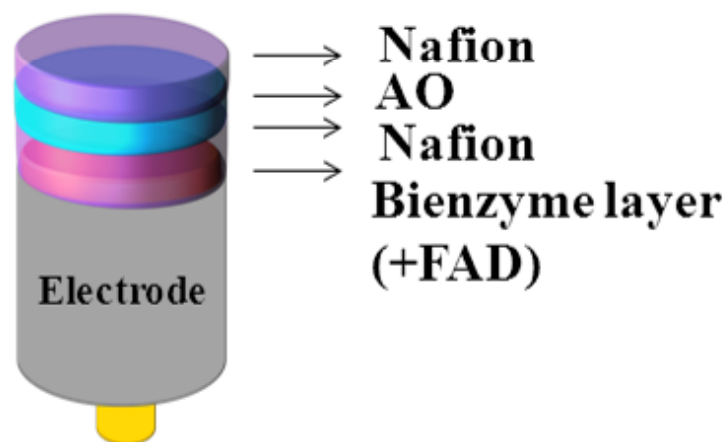
(B)

Fig. 1

Fig. 2

H₂O   O₂

Dehydro-
ascorbate

AO

AA

O₂   DAAs   DAAs

DAAO

Products

Interferents

H₂O₂

Electrode

PVI[Fe(CN)₅]³⁻

POD

AO

Nafion

H₂O

PVI[Fe(CN)₅]²⁻

Bienzyme layer

PVI[Fe(CN)₅]

Nafion
AO
Nafion
Bienzyme layer
(+FAD)

Electrode
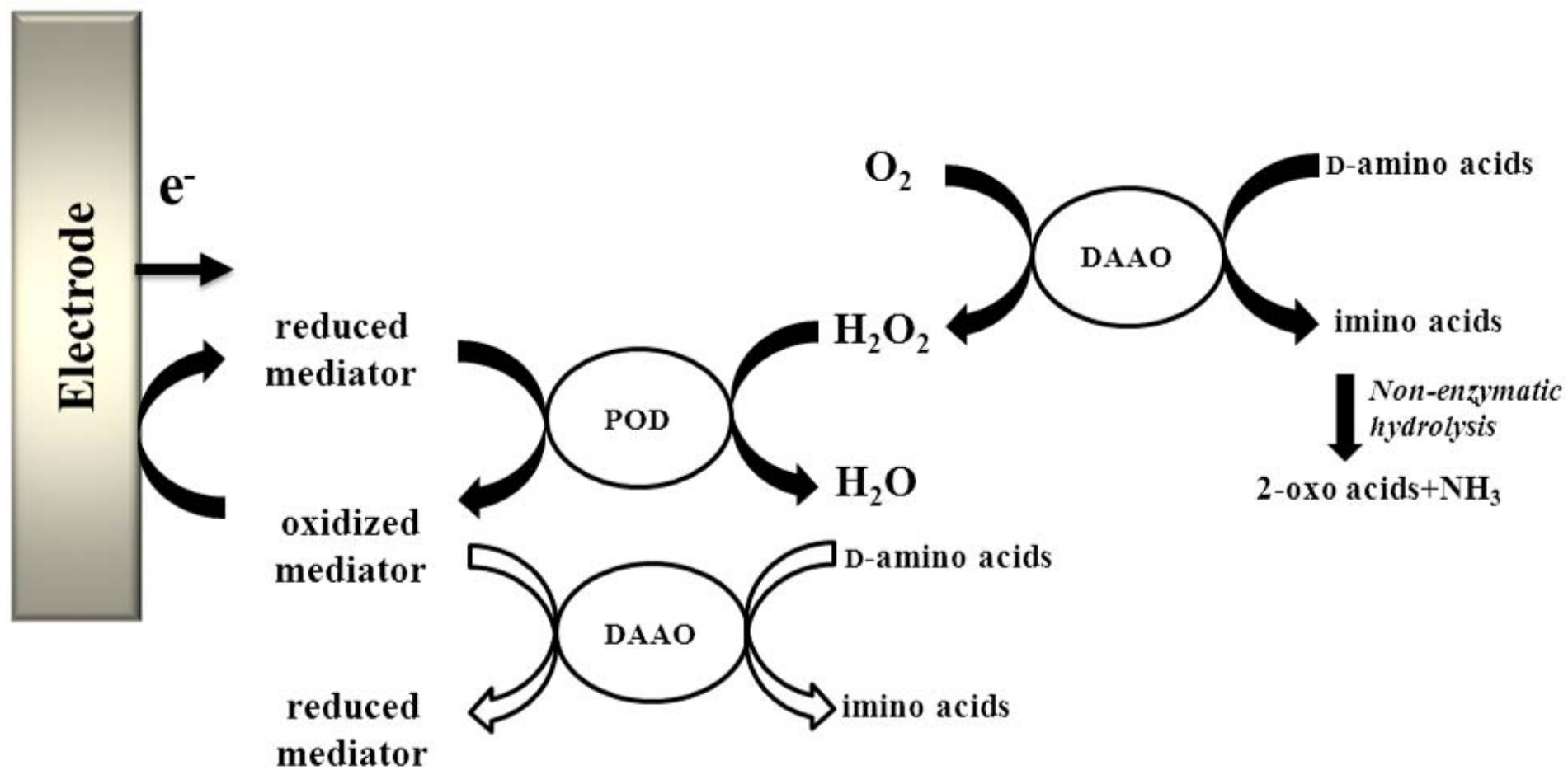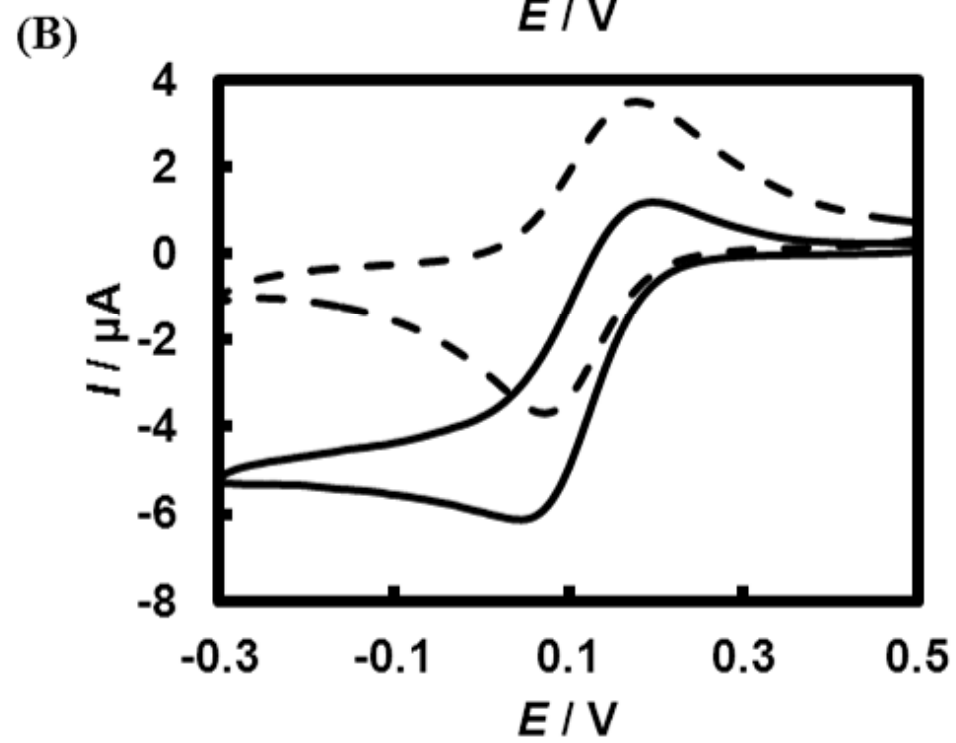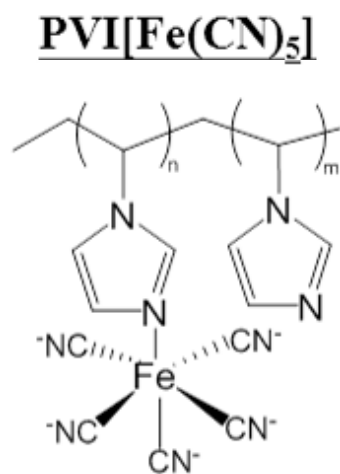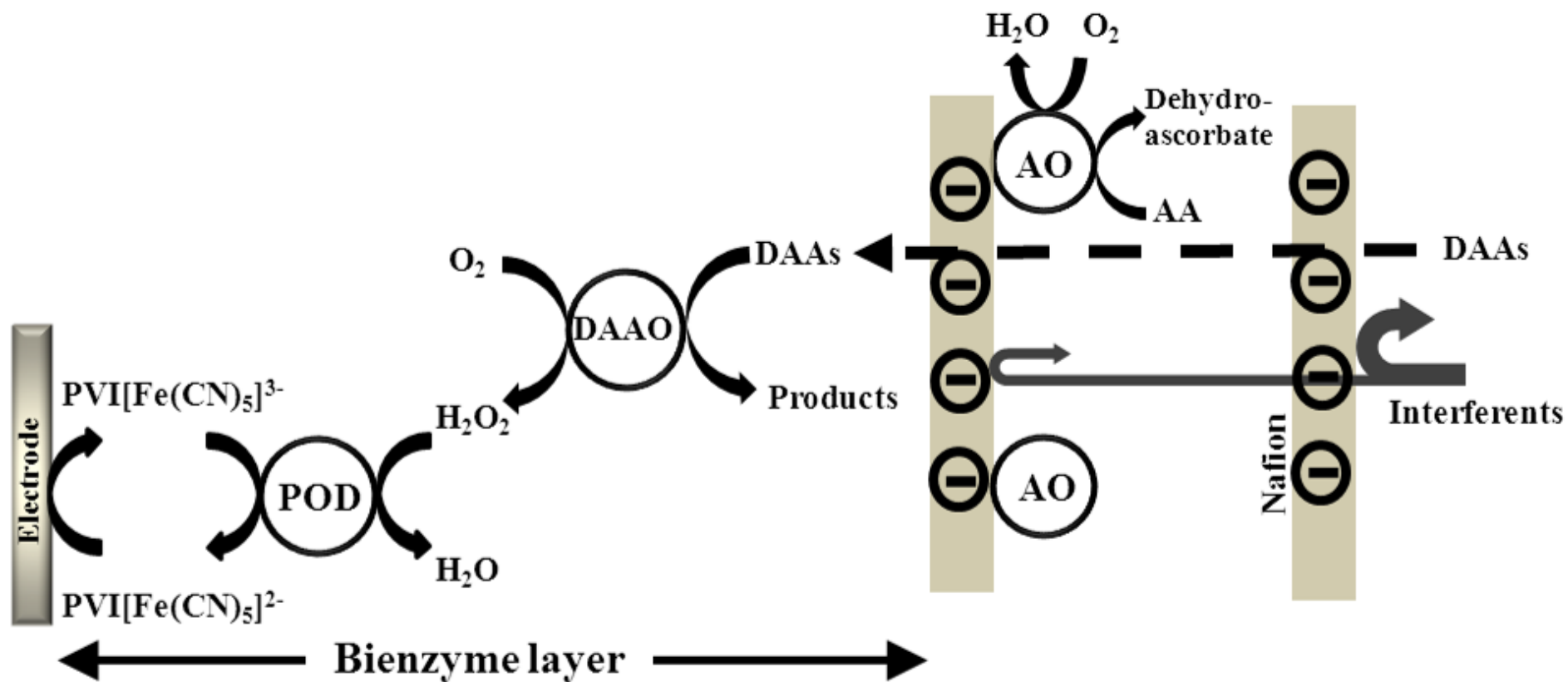
Scheme 2

Supplementary Data

Sensitive D-Amino Acid Biosensor Based on Oxidase/Peroxidase

System Mediated by Pentacyanoferrate-bound Polymer

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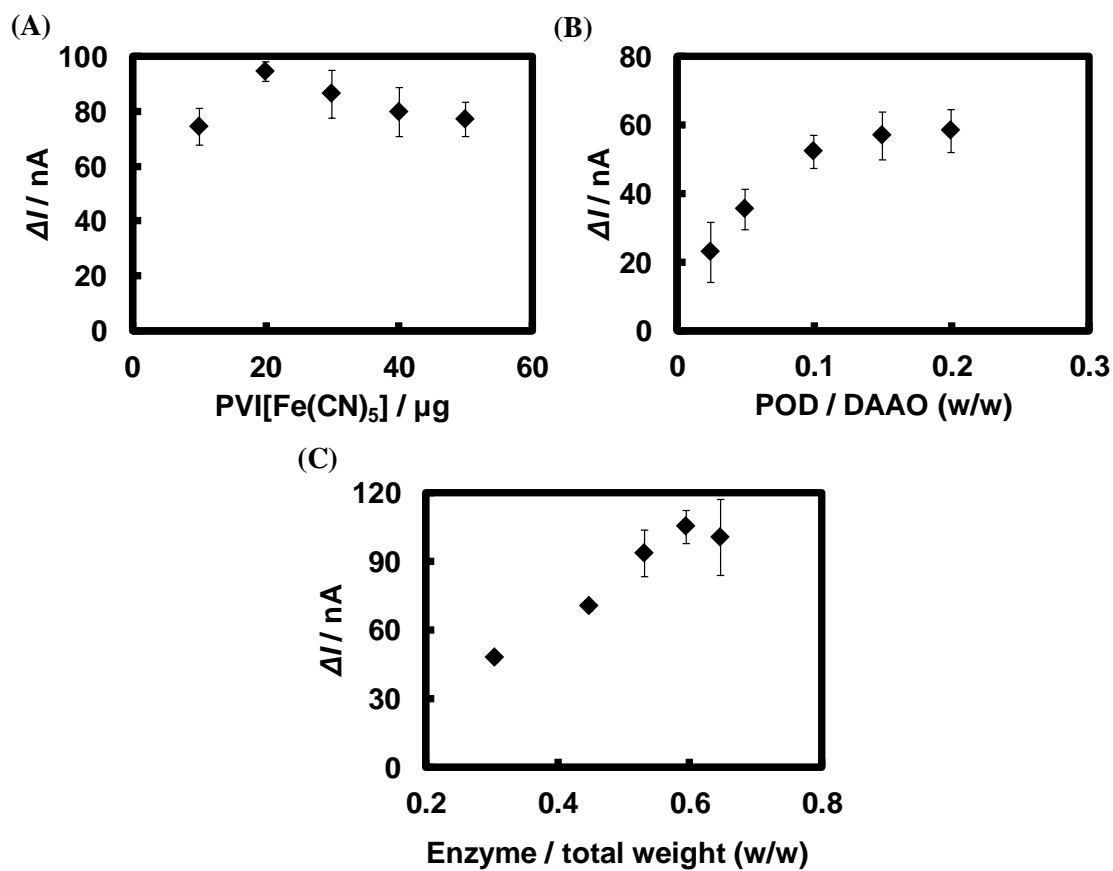
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Figure S1 Dependence of the amperometric response on the (A) amount of PVI[Fe(CN)$_5$], (B) the weight ratio of POD to DAAO and (C) the weight ratio of the enzymes to the total weight in the detection of 5 μM D-alanine at –0.1 V. All experimental solutions contain 40 μM FAD.
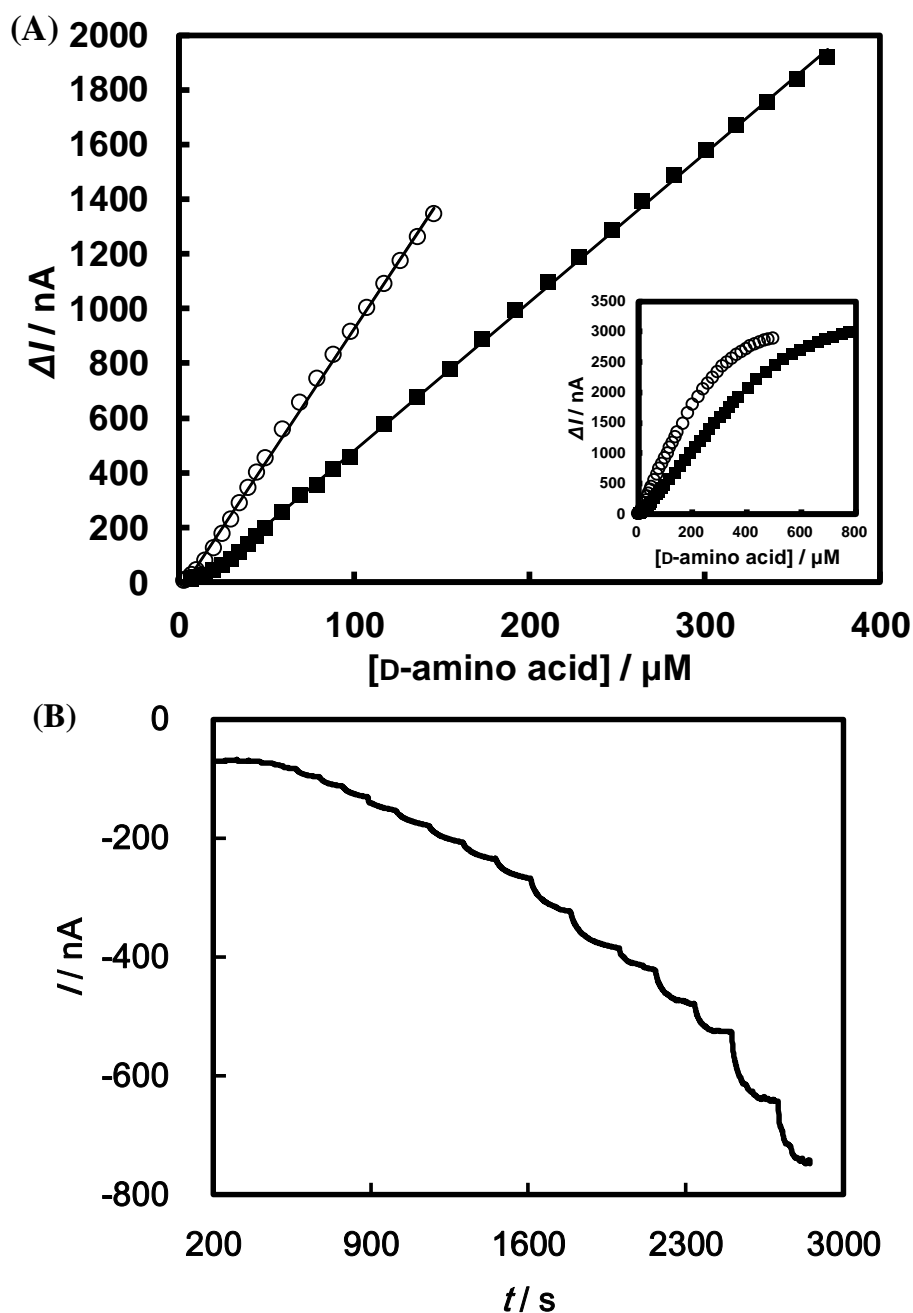
Figure S2 (A) Dependence of the amperometric response on the concentration of D-alanine (open circle) and D-serine (closed square) at –0.1V. Current response (nA) = 9.7 (nA μM^{-1})×[D-alanine] (μM)−40.7 (nA), R^2=0.999, and Current response (nA) = 5.4 (nA μM^{-1})×[D-serine] (μM)−62.3 (nA), R^2=0.999, respectively. The inset shows the current response in the wide range of the DAA concentration. (B) One part of typical current-time recordings for successive additions of D-serine (with different concentrations).
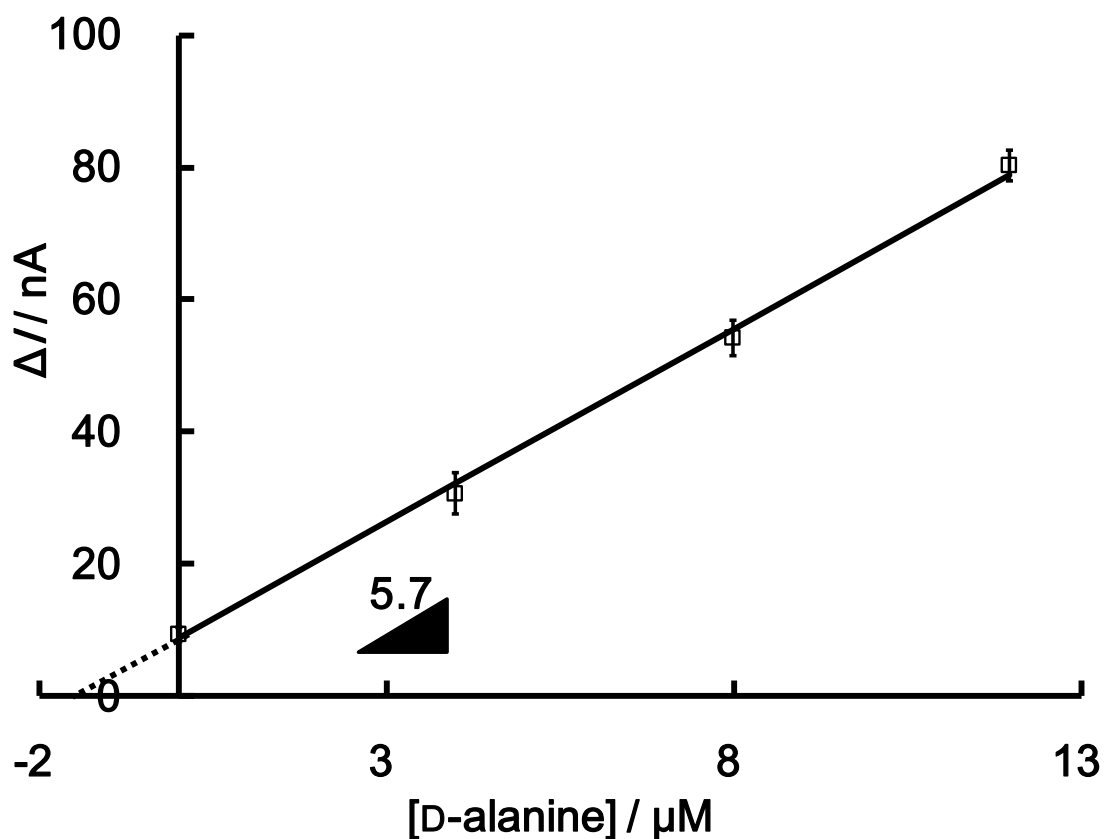
Figure S3 Standard addition measurements of a urine sample, which was 100 times diluted with 30 mM potassium phosphate buffer (pH 8.0). Current response (nA) = 5.8 ± 0.1 (nA μM^{-1})$\times$[D-alanine] (μM)+8.8 ± 0.7 (nA), R^2=0.998. The average values and the error bars are obtained from triplicate measurements. The slope of 5.7 nA μM^{-1} means the sensitivity of the bienzyme biosensor for the D-alanine detection in the solution without urine.