Amperometric Biosensor Based on Reductive H_2O_2 Detection Using Pentacyanoferrate-bound Polymer for Creatinine Determination

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Abstract

Pentacyanoferrate-bound poly(1-vinylimidazole) (PVI[Fe(CN)$_5$]) was selected as a mediator for amperometric creatinine determination based on the reductive H_2O_2 detection. Creatinine amidohydrolase (CNH), creatine amidohydrolase (CRH), sarcosine oxidase (SOD), peroxidase (POD) and PVI[Fe(CN)$_5$] were crosslinked with poly(ethylene glycol) diglycidyl ether (PEGDGE) on a glassy carbon (GC) electrode for a creatinine biosensor fabrication. Reduction current was monitored at −0.1 V in the presence of creatinine and O_2. It is revealed that PVI[Fe(CN)$_5$] is suitable as a mediator for a bioelectrocatalytic reaction of POD, since PVI[Fe(CN)$_5$] neither reacts with reactants nor works as an electron acceptor of SOD. The amounts of PVI[Fe(CN)$_5$], PEGDGE and enzymes were optimized towards creatinine detection. Nafion as a protecting film successfully prevented the enzyme layer from interference (uric acid and ascorbic acid). The detection limit and linear range in creatinine determination were 12 μM and 12−400 μM ($R^2 = 0.99$), respectively, which is applicable for urine creatinine test. The results of creatinine determination for four urine samples measured with this proposed method were compared with Jaffe method, and a good correlation was obtained between the results.

Keywords: Pentacyanoferrate-bound poly(1-vinylimidazole); Creatinine amidohydrolase; Reductive H_2O_2 detection; Peroxidase; Nafion; Urine creatinine test.

1. Introduction

Creatinine is the final product of creatine metabolism in muscle of mammals and is mainly filtered out of blood in kidneys. The creatinine levels are related to the state of renal function, thyroid malfunction and muscular disorders. The physiologically normal concentration ranges of creatinine in serum and urine are 40−150 μM and 2.5−23 mM, respectively; high creatinine level may result from renal impairment, while the low creatinine level indicates decreased muscle mass [1, 2]. The determination of urine creatinine is also important in other disease measurements since it is widely used as a calibration index for evaluating disease markers based on the constant excretion rate every day [3]. The current clinical determination of creatinine is based on colorimetric Jaffe reaction, which involves the formation of red products with picric acid in alkaline solution [4]. However, Jaffe method shows poor selectivity since it is affected by numerous metabolites containing carbonyl group found in biological samples, such as glucose, bilirubin and ascorbic acid [5, 6]. To increase specificity, creatinine deiminase (CD) has been utilized to generate ammonia for amperometric detection though it is interfered from endogenous ammonia [7, 8].

Rather than CD, creatinine amidohydrolase (CNH), creatine amidohydrolase (CRH) and sarcosine oxidase (SOD) have more widely been utilized for creatinine determination in amperometric method based on the detection of oxygen consumption or generated H_2O_2, which are so-called the first generation biosensor [9, 10]. The mechanism of creatinine reaction is shown as follows:

$$\text{Creatinine} + H_2O \xrightleftharpoons{\text{CNH}} \text{Creatine} \qquad (1)$$

$$\text{Creatine} + H_2O \xrightarrow{\text{CRH}} \text{Sarcosine} + \text{Urea} \qquad (2)$$

$$\text{Sarcosine} + H_2O + O_2 \xrightarrow{\text{SOD}} \text{Glycine} + \text{Formaldehyde} + H_2O_2 \qquad (3)$$

In the detection of oxygen consumption, the signal response is seriously influenced by the concentration of dissolved oxygen in samples and the diffusion rate of oxygen from the bulk solution to the surface of the working electrode. On the other hand, the direct electrooxidation of H_2O_2 requires high operation potential (+0.7 V vs. Ag|AgCl), which often accompanies the serious interference problem from other electroactive metabolites in physiological fluids.

In order to overcome this problem, the second generation biosensors have been evolved by using mediators to regenerate oxidized SOD (Eqs.4 and 5) [11]. Mediators shuttle electrons from the redox center of SOD to electrode (Eqs. 5 and 6), which provides higher signal response and lower operating potential.

$$\text{Sarcosine} + \text{SOD(FAD)} \rightarrow \text{Glysine} + \text{Formaldehyde} + \text{SOD}(\text{FADH}_2) \qquad (4)$$

$$\text{SOD}(\text{FADH}_2) + 2\text{Med}^{+}_{(ox)} \rightarrow \text{SOD(FAD)} + 2\text{Med}_{(red)} + 2H^{+} \qquad (5)$$

$$2\mathrm{Med}_{(\mathrm{red})} \rightarrow 2\mathrm{Med}^{+}{}_{(\mathrm{ox})} + 2e^{-} \quad (6)$$

Various kinds of redox mediators such as DCPIP, PMS, ferricyanide and hydroquinone were utilized for the SOD reaction [12, 13]. Nevertheless, the mediating capabilities of DCPIP, PMS and ferricyanide for SOD reaction are not good, and in our knowledge, most of quinones react with sarcosine to generate colored products. Furthermore, O_2 needs to be removed to avoid the competition with the mediator, which is difficult in practical analysis.

On the other hand, mediated biosensors (such as iron or osmium complexes) coupled with peroxidase (POD) allow the determination of H_2O_2 at low operating potentials around 0 V vs. Ag|AgCl, with high sensitivity, high stability, and elimination of the undesirable oxidation of interferents [14-16]. However, there is one thing to be concerned that mediators may react with both of oxidase and POD to cause a decrease in the electrochemical response of mediator reduction [17, 18]. Therefore, it is necessary to select an appropriate mediator with selective reactivity against POD alone.

In this study, pentacyanoferrate-bound poly(1-vinylimidazole) (PVI[$Fe(CN)_5$]) is selected as a mediator between POD and an electrode for creatinine determination considering its poor mediating capability against SOD. PVI[$Fe(CN)_5$] has been synthesized in our group for fast mediated electron transfer (MET) and immobilization of bilirubin oxidase for oxygen reduction [19]. This kind of electron-conducting hydrogel can covalently bound to enzymes, and it provides three-dimensional electrocatalysts which are not leachable but swollen in water to form stable redox hydrogels for MET between the redox center of enzymes and electrode [20]. The principle of creatinine detection is shown in Scheme 1. The three enzymes, POD and $PVI[Fe(CN)_5]$ were crosslinked with poly(ethylene glycol) diglycidyl ether (PEGDGE) on a glassy carbon (GC) electrode. Creatinine was hydrolyzed and oxidized to generate H_2O_2, then the reduction current of $PVI[Fe(CN)_5]$ was observed by the H_2O_2 reduction through POD. The catalytic effect of $PVI[Fe(CN)_5]$ on SOD and POD, electrode optimization, interference effect and the comparison with Jaffe method will be described.

2. Experimental

2.1 Reagents

2,2'-Azobisisobutyronitrile (AIBN), sodium pentacyanonitrosylferrate(III) dihydrate ($Na_2[Fe(CN)_5(NO)]\cdot 2H_2O$), sarcosine, creatine, creatinine, ascorbic acid (AA), uric acid (UA) and saturated picric acid solution were obtained from Wako Chem. Co. (Osaka, Japan). POD from horseradish (257 U mg^{-1}), SOD from *microorganism* (16.6 U mg^{-1}), CRH from *microorganism* (13 U mg^{-1}), and CNH from *microorganism* (258 U mg^{-1}) were purchased from Toyobo Co. (Osaka, Japan). 1-Vinylimidazole, PEGDGE and Nafion (5 wt% in mixture of lower aliphatic alcohols and water, contains 45% water) were from Sigma-Aldrich (USA). UA solution was prepared by dissolving in 10 mM NaOH, and the enzymes, substrates, AA and PEGDGE solutions were prepared using 100 mM phosphate buffer (pH 7.0). Other chemicals were of analytical grade and used as received. Urine samples were donated from healthy people.

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

Poly(1-vinylimidazole) (PVI) was synthesized according to the literature [20]. In brief, 6 mL of 1-vinylimidazole was mixed with 0.5 g of AIBN and was heated under stirring at 70 °C for 2 h in Ar. After cooling, the yellow precipitate was dissolved by adding methanol, followed by adding dropwise to acetone under strong stirring. White

PVI powder was obtained after filtering and drying. PVI[Fe(CN)$_5$] was then synthesized as reported [19]. Briefly, 200 mg of $Na_2[Fe(CN)_5(NO)]\cdot 2H_2O$ and 188 mg of PVI were dissolved in 50 mL of 0.6 M NaOH and refluxed at 65 °C for 24 h. The mixture was dialyzed against distilled water overnight, followed by centrifuging at 5000 *g* during 20 min 2 times to remove precipitate. The suspension was vacuum freeze-dried at −40 °C for 24 h to get light yellow PVI[Fe(CN)$_5$] powder. The stock solution of PVI[Fe(CN)$_5$] was prepared in a 10 mM phosphate buffer solution at pH 7.0.

2.3 Fabrication of enzymes and PVI[Fe(CN)$_5$]-modified electrode

The surface of a GC electrode (3 mm diameter, BAS) was polished with alumina powder, washed with distilled water and dried before use. Two μL of PVI[Fe(CN)$_5$], 1 μL of PEGDGE and 2 μL of enzyme solution were successively cast onto the surface of GC electrode 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 100 mM phosphate buffer (pH 7.0) for at least 30 min. For interference tests, 5 μL of 1% Nafion in ethanol was cast onto the surface of the proposed electrode and air-dried before immersing into buffer.

2.4 Electrochemical measurements

All electrochemical investigations were carried out in 100 mM phosphate buffer (pH 7.0) under moderate stirring at 25 °C with an electrochemical analyzer (BAS CV 50 W, BAS Inc., Japan). A platinum wire electrode and an Ag|AgCl sat. KCl electrode were used as the counter and the reference electrodes, respectively.

2.5 Creatinine determination by Jaffe method

This electro-enzymatic method was compared with spectrophotometric Jaffe method [21]. One hundred μL of urine sample or creatinine standard solution (0.5−2.5 mg mL^{-1}) prepared in a 10 mM HCl solution was added into a reagent solution containing 2 mL of saturated picric acid solution and 150 μL of 10 wt% NaOH. After the 10-min incubation at room temperature, 7.75 mL of distilled water was added into the resulting solution for 5 min, and the absorbance at $\lambda = 520$ nm was measured with a spectrophotometer (MultiSpec-1500, Shimadzu Co., Japan).

3. Results and discussion

3.1 Catalytic effect of PVI[Fe(CN)$_5$]on SOD and POD

In oxidase/peroxidase bienzyme system, mediators oxidized in the POD reaction may also be reduced by receiving the electron from the reduced oxidase generated in the substrate oxidation, which interferes with the detection of mediator reduction on the electrode. To evaluate the mediating effect for mediator selection, PVI[Fe(CN)$_5$] and PVI[Os(4,4'-dimethyl-2,2'-bipyridine)$_2$Cl] (PVI[Os(dmebpy)$_2$Cl]), which was synthesized according to the literature [22, 23], were used to investigate the interactions with SOD and POD. The cyclic voltammetric responses of SOD/POD-PVI[Fe(CN)$_5$]-modified GC electrode and SOD-PVI[Fe(CN)$_5$]-modified GC electrode were shown in Fig. 1. In Fig. 1A, PVI[Fe(CN)$_5$] did not mediate the SOD reaction, while the catalytic reduction current from POD reaction was clearly observed (Fig. 1B).

On the other hand, PVI[Os(dmebpy)$_2$Cl] synthesized according to the literature [22, 23] reacted with SOD; the catalytic oxidation current of creatinine was obtained (Fig. 1C). Therefore, in the cyclic voltammogram of SOD/POD-PVI[Os(dmebpy)$_2$Cl] electrode, the catalytic reduction current from POD reaction was hardly observed as shown in Fig. 1D.

The reason which causes the difference in the reactivity between PVI[Fe(CN)$_5$] and PVI[Os(dmebpy)$_2$Cl] can be explained as follows. Originally, the meditating capability of hexacyanoferrate ion on SOD reaction is not good because the flavin adenine dinucleotide (FAD), the redox center of SOD, locates in hydrophobic surroundings; hexacyanoferrate with negatively charged ligands would be difficult to enter into the deeply-buried FAD due to the electrostatic repulsion [24]. After binding pentacyanoferrate with PVI, it may become more difficult to enter the active site of SOD due to the increased charge density and fixation. For this reason, there is no mediating effect of PVI[Fe(CN)$_5$] on the SOD reaction. On the other hand, Os(dmebpy)$_2$Cl is more hydrophobic than pentacyanoferrate, which decreases the difficulty in entering the active site of the oxidase. In POD reaction, both of the polymers can transfer electrons to the protoheme, the redox center of POD. A reasonable explanation is that the location of protoheme is near the surface of POD, and the size of POD is smaller than that of SOD, which shortens the distance between the mediator and the redox center [25]. Therefore, it is easier for PVI[Fe(CN)$_5$] to react with POD than with SOD. Based on the results, PVI[Fe(CN)$_5$] is suitable as a mediator for the SOD/POD bienzyme system.

3.2 Optimization of enzymes and PVI[Fe(CN)$_5$]-modified electrode

Figure 2 shows the effect of the PVI[Fe(CN)$_5$] amount fabricated with the four enzymes on the GC electrode. Over 30 μg of PVI[Fe(CN)$_5$], the amperometric response did not vary dramatically. However, with a large amount of PVI[Fe(CN)$_5$], the longer time was needed to get the steady state (e.g., 600 s for one injection for the electrode containing 50 μg of PVI[Fe(CN)$_5$] while 300 s for the electrode containing 30 μg of PVI[Fe(CN)$_5$]). It indicates that the thick film of the polymer increases the difficulty of the substrate permeation. Considering the current response and the time to reach the steady state, 30 μg of PVI[Fe(CN)$_5$] was selected for the following experiments.

The weight percentage of PEGDGE was then examined in the range from 2.5% to 38.8% of the total weight of the cast on the electrode. The time to reach the steady state increased with the increase in the percentage of PEGDGE above 11.2%, while the magnitude of the current responses did not change significantly (data not shown). The high percentage of PEGDGE increases the rigidness of the polymer film to result in the poor permeability of the substrate. Based on the result, the percentage of PEGDGE was optimized to be 11.2%.

Since the enzyme composition of POD and SOD may affect the biosensing performance, the effect of POD/SOD ratio on the current response for creatinine detection was also examined in the range from 0.15 to 1 (w/w). Figure 3 shows that the highest current response was obtained at the ratio of 0.2 (5 μg of POD and 20 μg of SOD) for the detection of 100 μM creatinine, and the current response decreased gradually with an increase in the ratio of POD to SOD. The ratio of POD to SOD was therefore optimized to be 0.2.

The amounts of the four enzymes were then determined as follows: 1.29 U of POD, 0.42 U of SOD, 0.26 U of CRH and 1.29 U of CNH by considering the effect of the total weight of the enzyme on the current response.

3.3 Interference effect

The creatinine biosensor based on the reductive H_2O_2 detection at a low operating potential (−0.1 V vs. Ag|AgCl) minimizes the undesirable oxidation of electroactive interference in physiological fluids. However, some interferents such as AA may still react with POD, since they act as electron donors for the H_2O_2 reduction [26].

$$\text{interferents} + H_2O_2 \xrightarrow{\text{POD}} \text{oxidized interferents} + 2H_2 \qquad (7)$$

The reaction of the interferents with H_2O_2 catalyzed by POD (Eq. 7) decreases the H_2O_2 concentration produced in the oxidase reaction, which causes the underestimation of the creatinine concentration. To eliminate the interference, negatively-charged Nafion was utilized as a protecting film on the top of the enzymes-PVI[Fe(CN)$_5$]-modified electrode to exclude anionic species such as AA and UA. The interference effect on the amperometric response measured with the proposed electrode covered with and without Nafion film is shown in Fig. 4. For the detection of 150 μM creatinine, the amperometric response with the Nafion-coated electrode was smaller than that measured the electrode without Nafion film because of the inhibition of the mass transfer. However, the interference effect was eliminated by the protection of Nafion film, while the current responses of 150 μM UA and 10 μM AA were observed at the electrode without Nafion film. In the case of urine, the normal concentrations of creatinine and UA are in the same level [27], and the concentration of creatinine is about thirty times higher than that of AA [28]. This means that Nafion used as a protecting film satisfies the creatinine determination in real urine samples.

Internal creatine in urine might also interfere with creatinine determination since it reacts with CRH immobilized on the enzymes-PVI[Fe(CN)$_5$]-modified electrode to result in the overestimation of creatinine. In the case of urine, the excretion rate of creatinine is about twenty times higher than that of creatine [29]. In our experiment, as the concentration ratio of creatine to creatinine is 6.7% (10 μM creatine/150 μM creatinine), the signal ratio of creatine to creatinine is only about 2% (data not shown) though H_2O_2 generation from creatine requires fewer enzymatic steps than that from creatinine. It can be described that in neutral pH, creatinine is positively charged while creatine is a zwitterion, therefore creatinine is easier to penetrate the negatively charged Nafion film into the enzyme layer to get a larger amperometric response [30]. As a result, the internal creatine in urine does not significantly affect the creatinine determination in our method.

3.4 Comparison with Jaffe method

The amperometric response for the optimized biosensing electrode at −0.1 V is presented in Fig. 5. The detection limit of creatinine is 12 μM and the linear range is from 12 to 400 μM ($R^2 = 0.99$), which is sufficient for urine sample test. This method was applied to the creatinine determination of urine from four donators and was compared with Jaffe method which is widely used in clinical diagnosis. In the electro-enzymatic method, 10 μL of urine sample was injected into 1 mL of 100 mM phosphate buffer (pH 7.0) for measurements. Table 1 shows the creatinine concentrations evaluated from the absorbance at $\lambda = 520$ nm measured by Jaffe method and the current response at −0.1 V measured by this proposed method, respectively. Numbers 1 and 2 are the urine samples which were donated by male, while No. 3 and 4 were donated by female. The data show that the concentration of urine creatinine in male is higher than that in female, which is consistent with the typical human reference ranges, and a good correlation was obtained between the two methods. The values measured by the electro-enzymatic method are lower than Jaffe method, most probably because other compounds in urine (UA or AA) caused positive interference in Jaffe method. The results also show that the creatinine concentrations of No. 1 and 2 measured by Jaffe method are similar with each other, while the value of No. 2 is smaller than that of No. 1 measured with this method. This seems to be resulted from a high concentration of interference in No. 2, which reacts with picric acid to overestimate the creatinine concentration in Jaffe method.

4. Conclusion

The creatinine biosensor based on the reductive H_2O_2 detection was successfully developed. Pentacyanoferrate-bound polymer is suitable as a mediator and appropriate for the enzyme immobilization in this biosensing system, since it only mediates the POD reaction but not against the SOD reaction. The interference effect was eliminated by the Nafion film and the proposed method is applicable for clinical diagnosis.

References

[1] T. Arndt, Forensic Sci.Int., 186 (2009) 48.

[2] U. Lad, S. Khokhar, G.M. Kale, Anal. Chem., 80 (2008) 7910.

[3] S. Narayanan, H.D. Appleton, Clin. Chem., 26 (1980) 1119.

[4] R.W. Bonsnes, H.H. Taussky, J. Biol. Chem., 158 (1945) 581.

[5] J.A. Weber, A.P. Vanzanten, Clin. Chem., 37 (1991) 695.

[6] M.H. Kroll, N.A. Roach, B. Poe, R.J. Elin, Clin. Chem., 33 (1987) 1129.

[7] M. Gutierrez, S. Alegret, M. del Valle, Biosens. Bioelectron., 23 (2008) 795.

[8] C.S. Rui, Y. Kato, K. Sonomoto, Biosens. Bioelectron., 9 (1994) 429.

[9] T. Tsuchida, K. Yoda, Clin. Chem., 29 (1983) 51.

[10] V.K. Nguyen, C.M. Wolff, J.L. Seris, J.P. Schwing, Anal. Chem., 63 (1991) 611.

[11] A.E.G. Cass, G. Davis, M.J. Green, H.A.O. Hill, J. Electroanal. Chem., 190 (1985) 117.

[12] A. Ramanavicius, Anal. Bioanal. Chem., 387 (2007) 1899.

[13] I. Taniguchi, S. Miyamoto, S. Tomimura, F.M. Hawkridge, J. Electroanal. Chem., 240 (1988) 333.

[14] H. Sakai, R. Baba, K. Hashimoto, A. Fujishima, A. Heller, J. Phys. Chem., 99 (1995) 11896.

[15] H. Kinoshita, M. Torimura, K. Kano, T. Ikeda, Electroanal., 9 (1997) 1234.

[16] T. Ikeda, T. Shiraishi, M. Senda, Agric. Biol. Chem., 52 (1988) 3187.

[17] T.J. Ohara, M.S. Vreeke, F. Battaglini, A. Heller, Electroanal., 5 (1993) 825.

[18] R. Matsumoto, M. Mochizuki, K. Kano, T. Ikeda, Anal. Chem., 74 (2002) 3297.

[19] K. Ishibashi, S. Tsujimura, K. Kano, Electrochem., 76 (2008) 594.

[20] T.J. Ohara, R. Rajagopalan, A. Heller, Anal. Chem., 65 (1993) 3512.

[21] M. Bader, R. Wrbitzky, M. Blaszkewicz, C. van Thriel, Arch. Toxicol., 81 (2007) 335.

[22] S.R. Johnson, T.D. Westmoreland, J.V. Caspar, K.R. Barqawi, T.J. Meyer, Inorg. Chem., 27 (1988) 3195.

[23] T.J. Ohara, R. Rajagopalan, A. Heller, Anal. Chem., 66 (1994) 2451.

[24] J.J. Kulys, N.K. Cenas, Biochim. Biophys. Acta, 744 (1983) 57.

[25] N.C. Veitch, Phytochem., 65 (2004) 249.

[26] R. Maidan, A. Heller, Anal. Chem., 64 (1992) 2889.

[27] Y.G. Zuo, Y. Yang, Z. Zhu, W.S. He, Z. Aydin, Talanta, 83 (2011) 1707.

[28] M.P. Westerman, Y. Zhang, J.P. McConnell, P.A. Chezick, R. Neelam, S. Freels, L.S. Feldman, S. Allen, R. Baridi, L.E. Feldman, L.W.M. Fung, Am. J. Hematol., 65

(2000) 174.

[29] C. Beyer, Clin. Chem., 39 (1993) 1613.

[30] J.H. Shin, Y.S. Choi, H.J. Lee, S.H. Choi, J. Ha, I.J. Yoon, H. Nam, G.S. Cha, Anal. Chem., 73 (2001) 5965.

Figure captions:

Scheme 1

Reaction scheme of creatinine biosensor based on the reductive H_2O_2 detection mediated by PVI[Fe(CN)$_5$].

Figure 1

Cyclic voltammograms of (A) SOD-PVI[Fe(CN)$_5$] electrode, (B) SOD/POD-PVI[Fe(CN)$_5$] electrode, (C) SOD-PVI[Os(dmebpy)$_2$Cl] electrode and (D) SOD/POD-PVI[Os(dmebpy)$_2$Cl] electrode. (A) and (C) were measured in Ar-saturated solutions while (B) and (D) were measured in air-saturated solutions. The dash line represents the measurement in 10 mM phosphate buffer (pH 7.0) and the solid line represents the measurement in 5 mM sarcosine. Scan rate: 20 mV s^{-1}. Electrode conditions, SOD: 0.83 U, POD: 2.57 U, PEGDGE: 11 μg, PVI[Fe(CN)$_5$]: 30 μg, PVI[Os(dmebpy)$_2$Cl]: ca. 40 μg.

Figure 2

Dependence of the current response on the PVI[Fe(CN)$_5$] amount for the detection of 100 μM creatinine at –0.1 V. Electrode conditions, CNH: 1.29 U, CRH: 0.26 U, SOD:

0.33 U, POD: 1.03 U, PEGDGE: 20 μg.

Figure 3

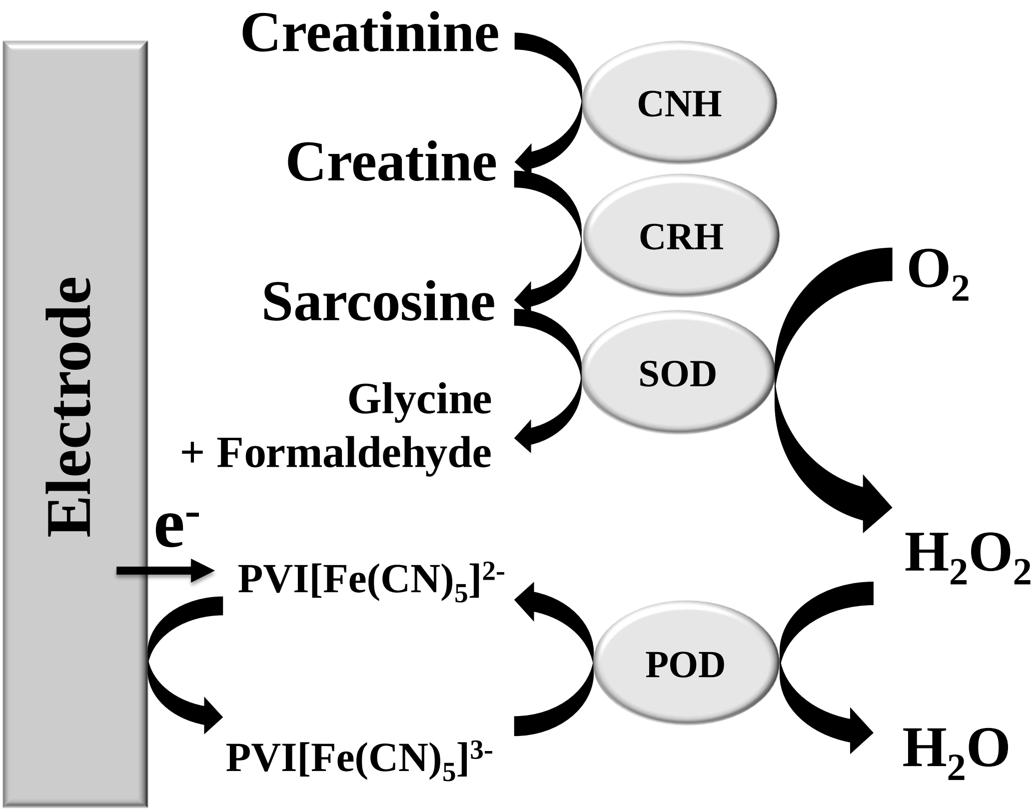
Dependence of the amperometric response on the weight ratio of POD to SOD for the detection of 100 μM creatinine at –0.1 V. Electrode conditions, CNH: 1.29 U, CRH: 0.26 U, SOD: 0.33 U (20 μg), PEGDGE: 11.2%, PVI[Fe(CN)$_5$]: 30 μg.

Figure 4

Amperometric responses of the proposed electrode without Nafion (solid line) and with 5 μL of 1% Nafion (dash line). CTN: 150 μM creatinine, UA: 150 μM, AA: 10 μM. The arrows indicate the injection time of the respective solutions. Electrode conditions, CNH: 1.29 U, CRH: 0.26 U, SOD: 0.42 U, POD: 1.29 U, PEGDGE: 11.2%, PVI[Fe(CN)$_5$]: 30 μg.

Figure 5

Dependence of the amperometric response on the creatinine concentration at –0.1V. Electrode conditions were the same as those in Fig. 4 with Nafion film.


Creatinine
CNH
Creatine
CRH
Sarcosine
SOD
Glycine
+ Formaldehyde
O_2
H_2O_2
Electrode
e^-
$PVI[Fe(CN)_5]^{2-}$
POD
$PVI[Fe(CN)_5]^{3-}$
H_2O

Scheme 1

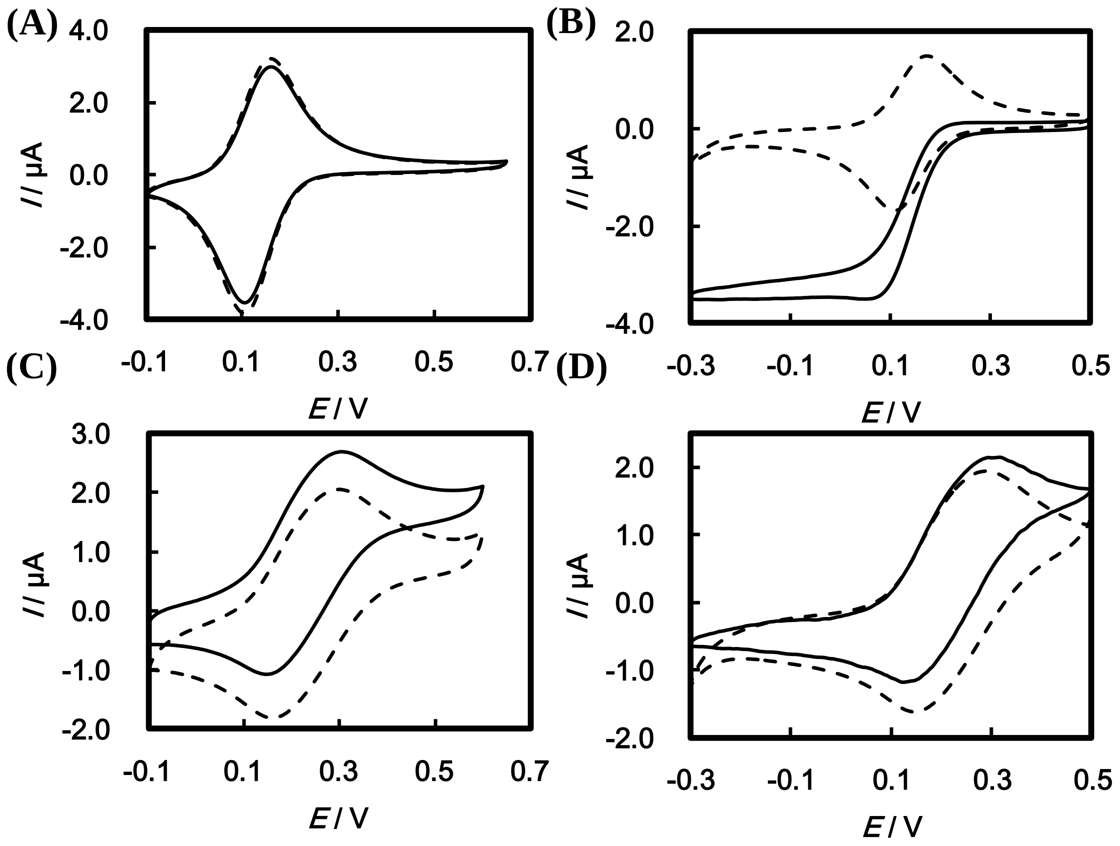

(A)
4.0
2.0
0.0
-2.0
-4.0
I / µA
-0.1
0.1
0.3
0.5
0.7
E / V
(B)
2.0
0.0
-2.0
-4.0
I / µA
-0.3
-0.1
0.1
0.3
0.5
E / V
(C)
3.0
2.0
1.0
0.0
-1.0
-2.0
I / µA
-0.1
0.1
0.3
0.5
0.7
E / V
(D)
2.0
1.0
0.0
-1.0
-2.0
I / µA
-0.3
-0.1
0.1
0.3
0.5
E / V

Figure 1

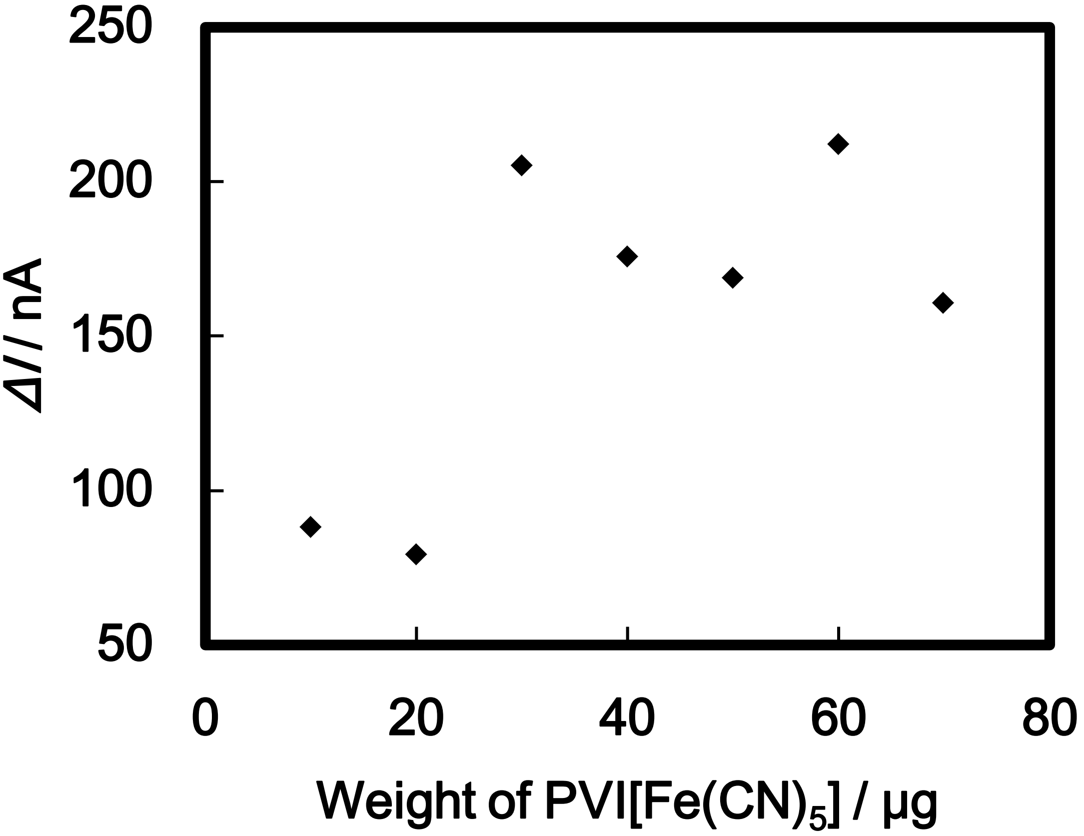


Figure 2

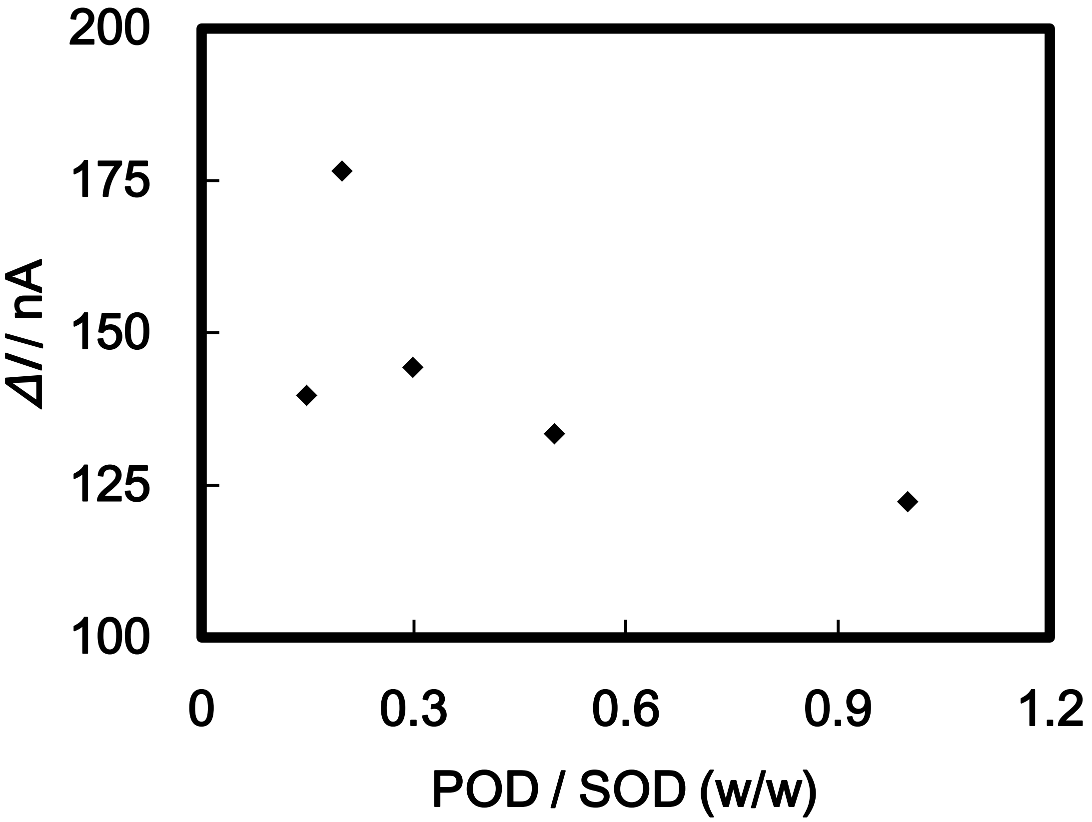
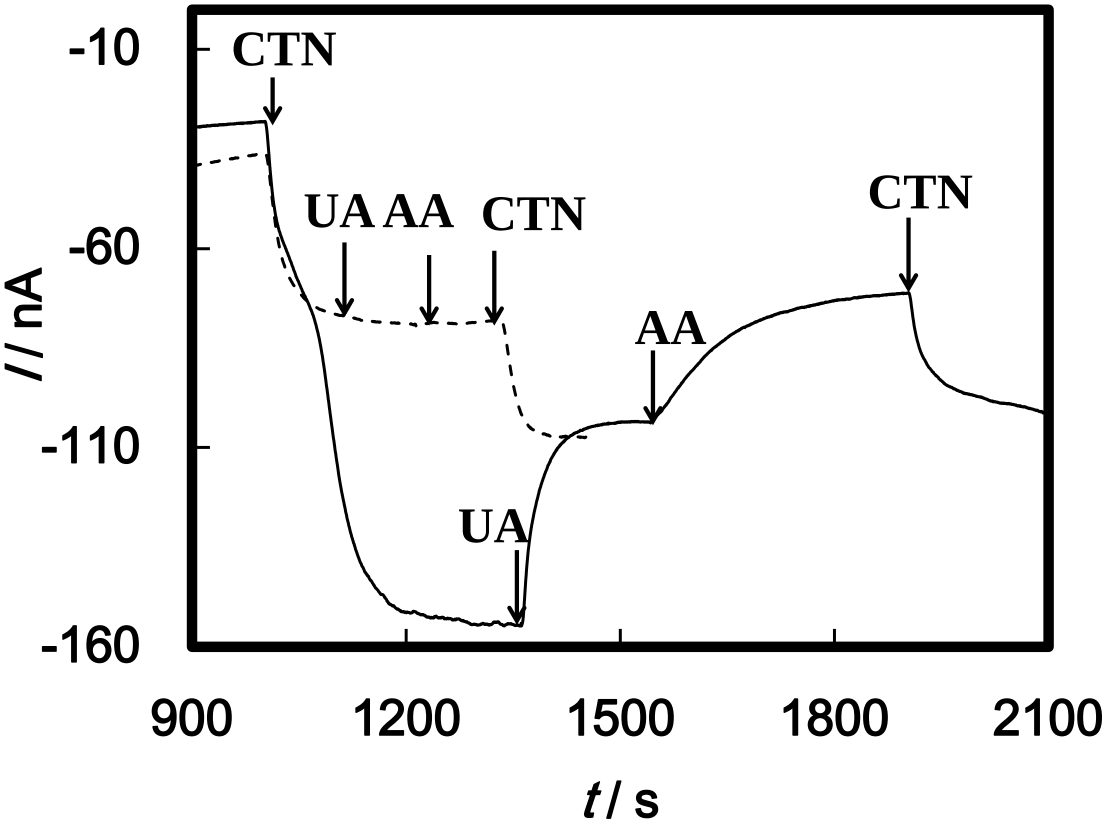


Figure 3


CTN
UA
AA
CTN
AA
UA
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-10
-60
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-160
I / nA
900
1200
1500
1800
2100
t / s

Figure 4

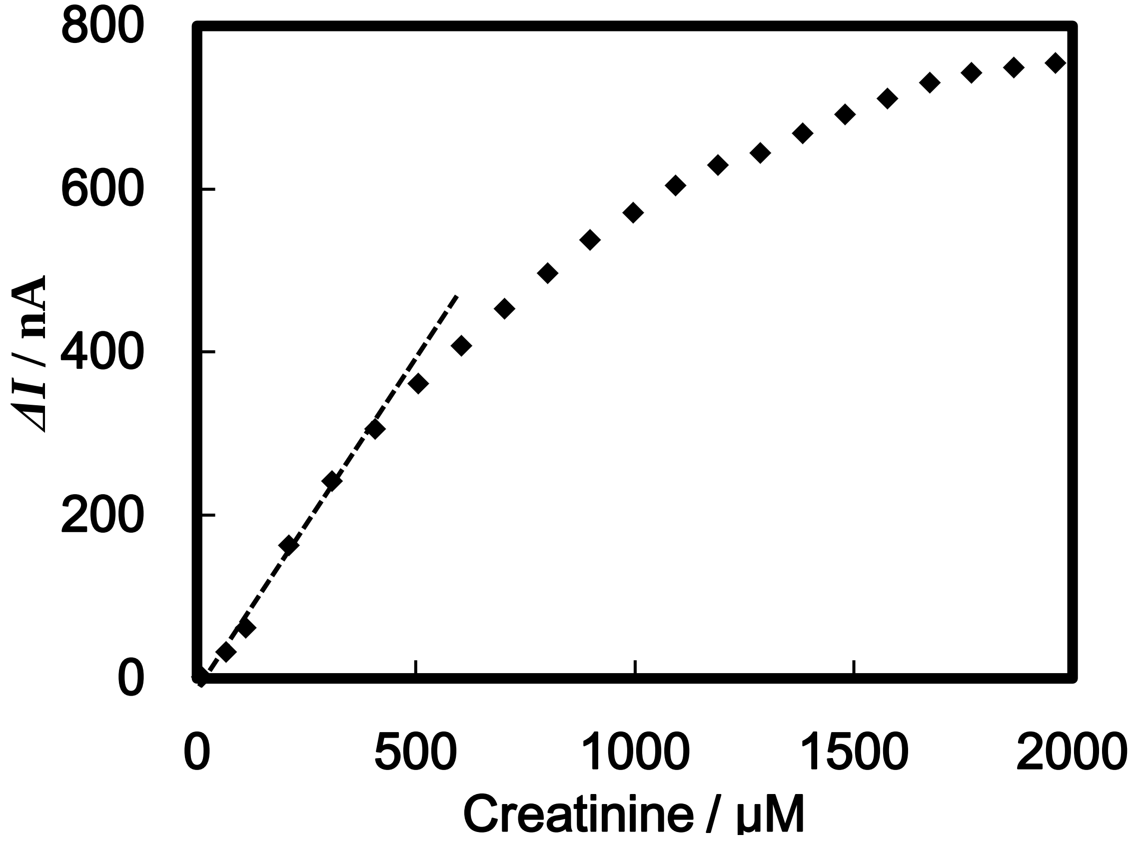

Figure 5

Table 1 Determination of creatinine from urine samples.

Number	**Creatinine / mM**	
	Jaffe method	**This method**
1	17.0±0.3	13.3±0.5
2	17.1±0.3	11.9±0.1
3	5.7±0.1	5.2±0.2
4	11.6±0.3	9.1±0.4

All data are the averages of triplicate experiments.