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Author(s)	Nieh, Chi-Hua; Tsujimura, Seiya; Shirai, Osamu; Kano, Kenji
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1 **Amperometric Biosensor Based on Reductive H_2O_2 Detection Using**

2 **Pentacyanoferrate-bound Polymer for Creatinine Determination**

3

4 Chi-Hua Nieh[1], Seiya Tsujimura[2], Osamu Shirai[1], Kenji Kano[1]*

5 1) Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University

6 Sakyo, Kyoto 606-8502, Japan, and 2) Division of Materials Science, Faculty of Pure

7 and Applied Sciences, University of Tsukuba 1-1-1 Tennodai, Tsukuba, Ibaraki,

8 305-8573, Japan

9

10 *Corresponding author

11 Tel: +81-75-753-6392

12 Fax: +81-75-753-6456

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

Abstract

14

15   Pentacyanoferrate-bound poly(1-vinylimidazole) ($PVI[Fe(CN)_5]$) was selected as a

16 mediator for amperometric creatinine determination based on the reductive H_2O_2

17 detection. Creatinine amidohydrolase (CNH), creatine amidohydrolase (CRH),

18 sarcosine oxidase (SOD), peroxidase (POD) and $PVI[Fe(CN)_5]$ were crosslinked with

19 poly(ethylene glycol) diglycidyl ether (PEGDGE) on a glassy carbon (GC) electrode for

20 a creatinine biosensor fabrication. Reduction current was monitored at -0.1 V in the

21 presence of creatinine and O_2. It is revealed that $PVI[Fe(CN)_5]$ is suitable as a mediator

22 for a bioelectrocatalytic reaction of POD, since $PVI[Fe(CN)_5]$ neither reacts with

23 reactants nor works as an electron acceptor of SOD. The amounts of $PVI[Fe(CN)_5]$,

24 PEGDGE and enzymes were optimized towards creatinine detection. Nafion as a

25 protecting film successfully prevented the enzyme layer from interference (uric acid and

26 ascorbic acid). The detection limit and linear range in creatinine determination were 12

27 μM and 12−400 μM ($R^2 = 0.99$), respectively, which is applicable for urine creatinine

28 test. The results of creatinine determination for four urine samples measured with this

29 proposed method were compared with Jaffe method, and a good correlation was
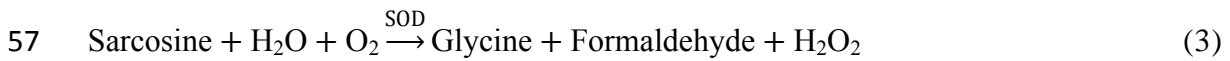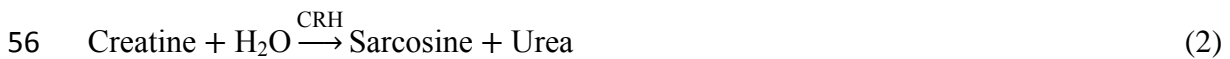
30 obtained between the results.

31

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].
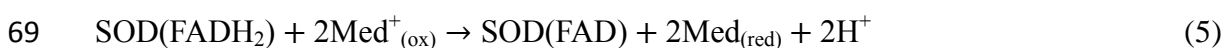
Rather than CD, creatinine amidohydrolase (CNH), creatine amidohydrolase (CRH) and sarcosine oxidase (SOD) have more widely been utilized for creatinine

4

52   determination in amperometric method based on the detection of oxygen consumption

53   or generated H_2O_2, which are so-called the first generation biosensor [9, 10]. The

54   mechanism of creatinine reaction is shown as follows:

55    $$\text{Creatinine} + H_2O \overset{\text{CNH}}{\underset{\longleftarrow}{\longrightarrow}} \text{Creatine} \tag{1}$$

56    $$\text{Creatine} + H_2O \overset{\text{CRH}}{\longrightarrow} \text{Sarcosine} + \text{Urea} \tag{2}$$

57    $$\text{Sarcosine} + H_2O + O_2 \overset{\text{SOD}}{\longrightarrow} \text{Glycine} + \text{Formaldehyde} + H_2O_2 \tag{3}$$

58   In the detection of oxygen consumption, the signal response is seriously influenced

59   by the concentration of dissolved oxygen in samples and the diffusion rate of oxygen

60   from the bulk solution to the surface of the working electrode. On the other hand, the

61   direct electrooxidation of H_2O_2 requires high operation potential (+0.7 V vs. Ag|AgCl),

62   which often accompanies the serious interference problem from other electroactive

63   metabolites in physiological fluids.

64   In order to overcome this problem, the second generation biosensors have been

65   evolved by using mediators to regenerate oxidized SOD (Eqs.4 and 5) [11]. Mediators

66   shuttle electrons from the redox center of SOD to electrode (Eqs. 5 and 6), which

67   provides higher signal response and lower operating potential.

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

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

70     $2Med_{(red)} \rightarrow 2Med^{+}_{(ox)} + 2e^{-}$ (6)

71   Various kinds of redox mediators such as DCPIP, PMS, ferricyanide and

72   hydroquinone were utilized for the SOD reaction [12, 13]. Nevertheless, the mediating

73   capabilities of DCPIP, PMS and ferricyanide for SOD reaction are not good, and in our

74   knowledge, most of quinones react with sarcosine to generate colored products.

75   Furthermore, O_2 needs to be removed to avoid the competition with the mediator, which

76   is difficult in practical analysis.

77   On the other hand, mediated biosensors (such as iron or osmium complexes)

78   coupled with peroxidase (POD) allow the determination of H_2O_2 at low operating

79   potentials around 0 V vs. Ag|AgCl, with high sensitivity, high stability, and elimination

80   of the undesirable oxidation of interferents [14-16]. However, there is one thing to be

81   concerned that mediators may react with both of oxidase and POD to cause a decrease

82   in the electrochemical response of mediator reduction [17, 18]. Therefore, it is

83   necessary to select an appropriate mediator with selective reactivity against POD alone.

84   In this study, pentacyanoferrate-bound poly(1-vinylimidazole) (PVI[Fe(CN)$_5$]) is

85   selected as a mediator between POD and an electrode for creatinine determination

86   considering its poor mediating capability against SOD. PVI[Fe(CN)$_5$] has been

87   synthesized in our group for fast mediated electron transfer (MET) and immobilization

88   of bilirubin oxidase for oxygen reduction [19]. This kind of electron-conducting

89   hydrogel can covalently bound to enzymes, and it provides three-dimensional

90   electrocatalysts which are not leachable but swollen in water to form stable redox

91   hydrogels for MET between the redox center of enzymes and electrode [20]. The

92   principle of creatinine detection is shown in Scheme 1. The three enzymes, POD and

93    $PVI[Fe(CN)_5]$ were crosslinked with poly(ethylene glycol) diglycidyl ether (PEGDGE)

94   on a glassy carbon (GC) electrode. Creatinine was hydrolyzed and oxidized to generate

95   H_2O_2, then the reduction current of $PVI[Fe(CN)_5]$ was observed by the H_2O_2 reduction

96   through POD. The catalytic effect of $PVI[Fe(CN)_5]$ on SOD and POD, electrode

97   optimization, interference effect and the comparison with Jaffe method will be

98   described.

99 **2. Experimental**

100 **2.1 Reagents**

101 2,2'-Azobisisobutyronitrile (AIBN), sodium pentacyanonitrosylferrate(III) dihydrate

102 $(Na_2[Fe(CN)_5(NO)] \cdot 2H_2O)$, sarcosine, creatine, creatinine, ascorbic acid (AA), uric acid

103 (UA) and saturated picric acid solution were obtained from Wako Chem. Co. (Osaka,

104 Japan). POD from horseradish (257 U mg^{-1}), SOD from *microorganism* (16.6 U mg^{-1}),

105 CRH from *microorganism* (13 U mg^{-1}), and CNH from *microorganism* (258 U mg^{-1})

106 were purchased from Toyobo Co. (Osaka, Japan). 1-Vinylimidazole, PEGDGE and

107 Nafion (5 wt% in mixture of lower aliphatic alcohols and water, contains 45% water)

108 were from Sigma-Aldrich (USA). UA solution was prepared by dissolving in 10 mM

109 NaOH, and the enzymes, substrates, AA and PEGDGE solutions were prepared using

110 100 mM phosphate buffer (pH 7.0). Other chemicals were of analytical grade and used

111 as received. Urine samples were donated from healthy people.

112 **2.2 Synthesis of PVI[Fe(CN)$_5$]**

113 Poly(1-vinylimidazole) (PVI) was synthesized according to the literature [20]. In

114 brief, 6 mL of 1-vinylimidazole was mixed with 0.5 g of AIBN and was heated under

115 stirring at 70 °C for 2 h in Ar. After cooling, the yellow precipitate was dissolved by

116 adding methanol, followed by adding dropwise to acetone under strong stirring. White

8

117 PVI powder was obtained after filtering and drying. PVI[Fe(CN)$_5$] was then synthesized

118 as reported [19]. Briefly, 200 mg of Na$_2$[Fe(CN)$_5$(NO)]·2H$_2$O and 188 mg of PVI were

119 dissolved in 50 mL of 0.6 M NaOH and refluxed at 65 °C for 24 h. The mixture was

120 dialyzed against distilled water overnight, followed by centrifuging at 5000 g during 20

121 min 2 times to remove precipitate. The suspension was vacuum freeze-dried at −40 °C

122 for 24 h to get light yellow PVI[Fe(CN)$_5$] powder. The stock solution of PVI[Fe(CN)$_5$]

123 was prepared in a 10 mM phosphate buffer solution at pH 7.0.

124 **2.3 Fabrication of enzymes and PVI[Fe(CN)$_5$]-modified electrode**

125 The surface of a GC electrode (3 mm diameter, BAS) was polished with alumina

126 powder, washed with distilled water and dried before use. Two μL of PVI[Fe(CN)$_5$], 1

127 μL of PEGDGE and 2 μL of enzyme solution were successively cast onto the surface of

128 GC electrode and well mixed with a syringe needle. The electrode was dried at 4 °C for

129 24 h. Before measurements, the proposed electrode was immersed into 100 mM

130 phosphate buffer (pH 7.0) for at least 30 min. For interference tests, 5 μL of 1% Nafion

131 in ethanol was cast onto the surface of the proposed electrode and air-dried before

132 immersing into buffer.

133 **2.4 Electrochemical measurements**

134   All electrochemical investigations were carried out in 100 mM phosphate buffer (pH

135   7.0) under moderate stirring at 25 °C with an electrochemical analyzer (BAS CV 50 W,

136   BAS Inc., Japan). A platinum wire electrode and an Ag|AgCl sat. KCl electrode were

137   used as the counter and the reference electrodes, respectively.

138   **2.5 Creatinine determination by Jaffe method**

139   This electro-enzymatic method was compared with spectrophotometric Jaffe method

140   [21]. One hundred μL of urine sample or creatinine standard solution (0.5−2.5 mg mL^{-1})

141   prepared in a 10 mM HCl solution was added into a reagent solution containing 2 mL of

142   saturated picric acid solution and 150 μL of 10 wt% NaOH. After the 10-min incubation

143   at room temperature, 7.75 mL of distilled water was added into the resulting solution for

144   5 min, and the absorbance at $\lambda = 520$ nm was measured with a spectrophotometer

145   (MultiSpec-1500, Shimadzu Co., Japan).

146 **3.   Results and discussion**

147 **3.1 Catalytic effect of PVI[Fe(CN)$_5$]on SOD and POD**

148   In oxidase/peroxidase bienzyme system, mediators oxidized in the POD reaction

149 may also be reduced by receiving the electron from the reduced oxidase generated in the

150 substrate oxidation, which interferes with the detection of mediator reduction on the

151 electrode. To evaluate the mediating effect for mediator selection, PVI[Fe(CN)$_5$] and

152 PVI[Os(4,4'-dimethyl-2,2'-bipyridine)$_2$Cl]   (PVI[Os(dmebpy)$_2$Cl]),   which   was

153 synthesized according to the literature [22, 23], were used to investigate the interactions

154 with   SOD   and   POD.   The   cyclic   voltammetric   responses   of

155 SOD/POD-PVI[Fe(CN)$_5$]-modified GC electrode and SOD-PVI[Fe(CN)$_5$]-modified GC

156 electrode were shown in Fig. 1. In Fig. 1A, PVI[Fe(CN)$_5$] did not mediate the SOD

157 reaction, while the catalytic reduction current from POD reaction was clearly observed

158 (Fig. 1B).

159   On the other hand, PVI[Os(dmebpy)$_2$Cl] synthesized according to the literature [22,

160 23] reacted with SOD; the catalytic oxidation current of creatinine was obtained (Fig.

161 1C). Therefore, in the cyclic voltammogram of SOD/POD-PVI[Os(dmebpy)$_2$Cl]

162 electrode, the catalytic reduction current from POD reaction was hardly observed as

163 shown in Fig. 1D.

164   The reason which causes the difference in the reactivity between PVI[Fe(CN)$_5$]

165   and PVI[Os(dmebpy)$_2$Cl] can be explained as follows. Originally, the meditating

166   capability of hexacyanoferrate ion on SOD reaction is not good because the flavin

167   adenine dinucleotide (FAD), the redox center of SOD, locates in hydrophobic

168   surroundings; hexacyanoferrate with negatively charged ligands would be difficult to

169   enter into the deeply-buried FAD due to the electrostatic repulsion [24]. After binding

170   pentacyanoferrate with PVI, it may become more difficult to enter the active site of

171   SOD due to the increased charge density and fixation. For this reason, there is no

172   mediating effect of PVI[Fe(CN)$_5$] on the SOD reaction. On the other hand,

173   Os(dmebpy)$_2$Cl is more hydrophobic than pentacyanoferrate, which decreases the

174   difficulty in entering the active site of the oxidase. In POD reaction, both of the

175   polymers can transfer electrons to the protoheme, the redox center of POD. A

176   reasonable explanation is that the location of protoheme is near the surface of POD, and

177   the size of POD is smaller than that of SOD, which shortens the distance between the

178   mediator and the redox center [25]. Therefore, it is easier for PVI[Fe(CN)$_5$] to react with

179   POD than with SOD. Based on the results, PVI[Fe(CN)$_5$] is suitable as a mediator for

180   the SOD/POD bienzyme system.

181   **3.2 Optimization of enzymes and PVI[Fe(CN)$_5$]-modified electrode**

182   Figure 2 shows the effect of the PVI[Fe(CN)$_5$] amount fabricated with the four

183   enzymes on the GC electrode. Over 30 μg of PVI[Fe(CN)$_5$], the amperometric response

184   did not vary dramatically. However, with a large amount of PVI[Fe(CN)$_5$], the longer

185   time was needed to get the steady state (e.g., 600 s for one injection for the electrode

186   containing 50 μg of PVI[Fe(CN)$_5$] while 300 s for the electrode containing 30 μg of

187   PVI[Fe(CN)$_5$]). It indicates that the thick film of the polymer increases the difficulty of

188   the substrate permeation. Considering the current response and the time to reach the

189   steady state, 30 μg of PVI[Fe(CN)$_5$] was selected for the following experiments.

190   The weight percentage of PEGDGE was then examined in the range from 2.5% to

191   38.8% of the total weight of the cast on the electrode. The time to reach the steady state

192   increased with the increase in the percentage of PEGDGE above 11.2%, while the

193   magnitude of the current responses did not change significantly (data not shown). The

194   high percentage of PEGDGE increases the rigidness of the polymer film to result in the

195   poor permeability of the substrate. Based on the result, the percentage of PEGDGE was

196   optimized to be 11.2%.

197   Since the enzyme composition of POD and SOD may affect the biosensing

198   performance, the effect of POD/SOD ratio on the current response for creatinine

199   detection was also examined in the range from 0.15 to 1 (w/w). Figure 3 shows that the

13

200 highest current response was obtained at the ratio of 0.2 (5 μg of POD and 20 μg of

201 SOD) for the detection of 100 μM creatinine, and the current response decreased

202 gradually with an increase in the ratio of POD to SOD. The ratio of POD to SOD was

203 therefore optimized to be 0.2.

204 The amounts of the four enzymes were then determined as follows: 1.29 U of POD,

205 0.42 U of SOD, 0.26 U of CRH and 1.29 U of CNH by considering the effect of the

206 total weight of the enzyme on the current response.

207 **3.3 Interference effect**

208 The creatinine biosensor based on the reductive H_2O_2 detection at a low operating

209 potential (-0.1 V vs. Ag|AgCl) minimizes the undesirable oxidation of electroactive

210 interference in physiological fluids. However, some interferents such as AA may still

211 react with POD, since they act as electron donors for the H_2O_2 reduction [26].

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

213 The reaction of the interferents with H_2O_2 catalyzed by POD (Eq. 7) decreases the

214 H_2O_2 concentration produced in the oxidase reaction, which causes the underestimation

215 of the creatinine concentration. To eliminate the interference, negatively-charged Nafion

216 was utilized as a protecting film on the top of the enzymes-PVI[Fe(CN)$_5$]-modified

217 electrode to exclude anionic species such as AA and UA. The interference effect on the

218   amperometric response measured with the proposed electrode covered with and without

219   Nafion film is shown in Fig. 4. For the detection of 150 μM creatinine, the

220   amperometric response with the Nafion-coated electrode was smaller than that

221   measured the electrode without Nafion film because of the inhibition of the mass

222   transfer. However, the interference effect was eliminated by the protection of Nafion

223   film, while the current responses of 150 μM UA and 10 μM AA were observed at the

224   electrode without Nafion film. In the case of urine, the normal concentrations of

225   creatinine and UA are in the same level [27], and the concentration of creatinine is about

226   thirty times higher than that of AA [28]. This means that Nafion used as a protecting

227   film satisfies the creatinine determination in real urine samples.

228   Internal creatine in urine might also interfere with creatinine determination since it

229   reacts with CRH immobilized on the enzymes-PVI[Fe(CN)$_5$]-modified electrode to

230   result in the overestimation of creatinine. In the case of urine, the excretion rate of

231   creatinine is about twenty times higher than that of creatine [29]. In our experiment, as

232   the concentration ratio of creatine to creatinine is 6.7% (10 μM creatine/150 μM

233   creatinine), the signal ratio of creatine to creatinine is only about 2% (data not shown)

234   though H_2O_2 generation from creatine requires fewer enzymatic steps than that from

235   creatinine. It can be described that in neutral pH, creatinine is positively charged while

236   creatine is a zwitterion, therefore creatinine is easier to penetrate the negatively charged

237   Nafion film into the enzyme layer to get a larger amperometric response [30]. As a

238   result, the internal creatine in urine does not significantly affect the creatinine

239   determination in our method.

240   **3.4 Comparison with Jaffe method**

241   The amperometric response for the optimized biosensing electrode at −0.1 V is

242   presented in Fig. 5. The detection limit of creatinine is 12 μM and the linear range is

243   from 12 to 400 μM (R^2 = 0.99), which is sufficient for urine sample test. This method

244   was applied to the creatinine determination of urine from four donators and was

245   compared with Jaffe method which is widely used in clinical diagnosis. In the

246   electro-enzymatic method, 10 μL of urine sample was injected into 1 mL of 100 mM

247   phosphate buffer (pH 7.0) for measurements. Table 1 shows the creatinine

248   concentrations evaluated from the absorbance at $\lambda = 520$ nm measured by Jaffe method

249   and the current response at −0.1 V measured by this proposed method, respectively.

250   Numbers 1 and 2 are the urine samples which were donated by male, while No. 3 and 4

251   were donated by female. The data show that the concentration of urine creatinine in

252   male is higher than that in female, which is consistent with the typical human reference

253   ranges, and a good correlation was obtained between the two methods. The values

254 measured by the electro-enzymatic method are lower than Jaffe method, most probably

255 because other compounds in urine (UA or AA) caused positive interference in Jaffe

256 method. The results also show that the creatinine concentrations of No. 1 and 2

257 measured by Jaffe method are similar with each other, while the value of No. 2 is

258 smaller than that of No. 1 measured with this method. This seems to be resulted from a

259 high concentration of interference in No. 2, which reacts with picric acid to

260 overestimate the creatinine concentration in Jaffe method.

4. Conclusion

261

262   The creatinine biosensor based on the reductive H_2O_2 detection was successfully

263   developed. Pentacyanoferrate-bound polymer is suitable as a mediator and appropriate

264   for the enzyme immobilization in this biosensing system, since it only mediates the

265   POD reaction but not against the SOD reaction. The interference effect was eliminated

266   by the Nafion film and the proposed method is applicable for clinical diagnosis.

267 **References**

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306 **Figure captions:**

307 Scheme 1

308 Reaction scheme of creatinine biosensor based on the reductive H_2O_2 detection

309 mediated by $PVI[Fe(CN)_5]$.

310

311 Figure 1

312 Cyclic voltammograms of (A) $SOD-PVI[Fe(CN)_5]$ electrode, (B) SOD/POD-

313 $PVI[Fe(CN)_5]$ electrode, (C) $SOD-PVI[Os(dmebpy)_2Cl]$ electrode and (D) SOD/POD-

314 $PVI[Os(dmebpy)_2Cl]$ electrode. (A) and (C) were measured in Ar-saturated solutions

315 while (B) and (D) were measured in air-saturated solutions. The dash line represents the

316 measurement in 10 mM phosphate buffer (pH 7.0) and the solid line represents the

317 measurement in 5 mM sarcosine. Scan rate: 20 mV s^{-1}. Electrode conditions, SOD: 0.83

318 U, POD: 2.57 U, PEGDGE: 11 μg, $PVI[Fe(CN)_5]$: 30 μg, $PVI[Os(dmebpy)_2Cl]$: ca. 40

319 μg.

320

321 Figure 2

322 Dependence of the current response on the $PVI[Fe(CN)_5]$ amount for the detection of

323 100 μM creatinine at –0.1 V. Electrode conditions, CNH: 1.29 U, CRH: 0.26 U, SOD:

324 0.33 U, POD: 1.03 U, PEGDGE: 20 μg.

325

326 Figure 3

327 Dependence of the amperometric response on the weight ratio of POD to SOD for the

328 detection of 100 μM creatinine at −0.1 V. Electrode conditions, CNH: 1.29 U, CRH:

329 0.26 U, SOD: 0.33 U (20 μg), PEGDGE: 11.2%, PVI[Fe(CN)$_5$]: 30 μg.

330

331 Figure 4

332 Amperometric responses of the proposed electrode without Nafion (solid line) and with

333 5 μL of 1% Nafion (dash line). CTN: 150 μM creatinine, UA: 150 μM, AA: 10 μM. The

334 arrows indicate the injection time of the respective solutions. Electrode conditions,

335 CNH: 1.29 U, CRH: 0.26 U, SOD: 0.42 U, POD: 1.29 U, PEGDGE: 11.2%,

336 PVI[Fe(CN)$_5$]: 30 μg.

337

338 Figure 5

339 Dependence of the amperometric response on the creatinine concentration at −0.1V.

340 Electrode conditions were the same as those in Fig. 4 with Nafion film.

Creatinine

CNH

Creatine

CRH

O$_2$

Sarcosine

SOD

Glycine + Formaldehyde

H$_2$O$_2$

Electrode
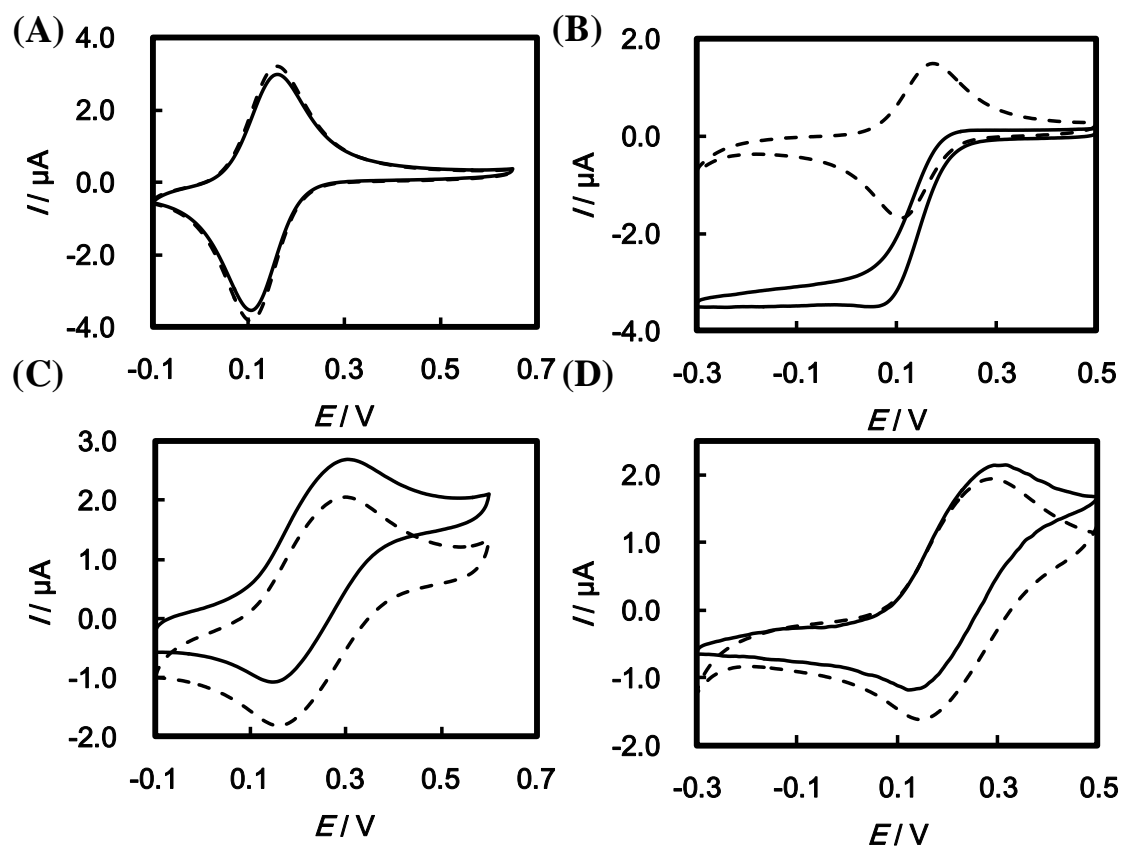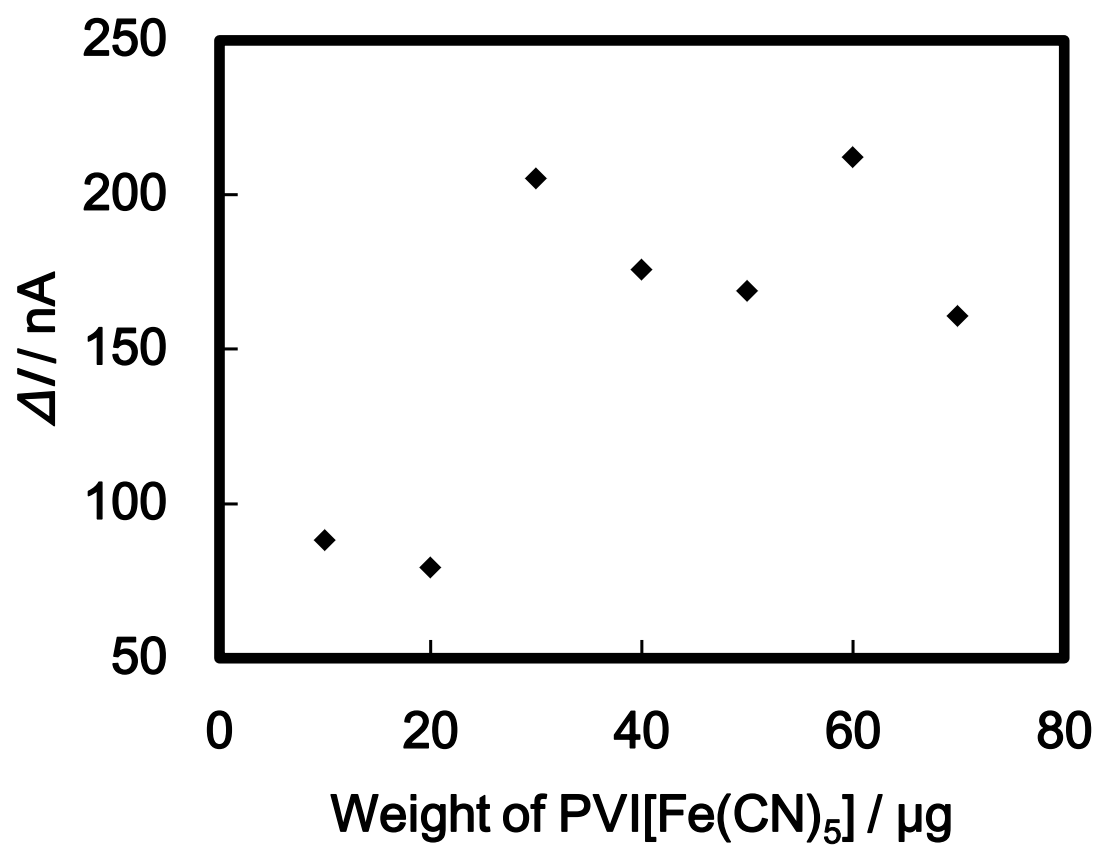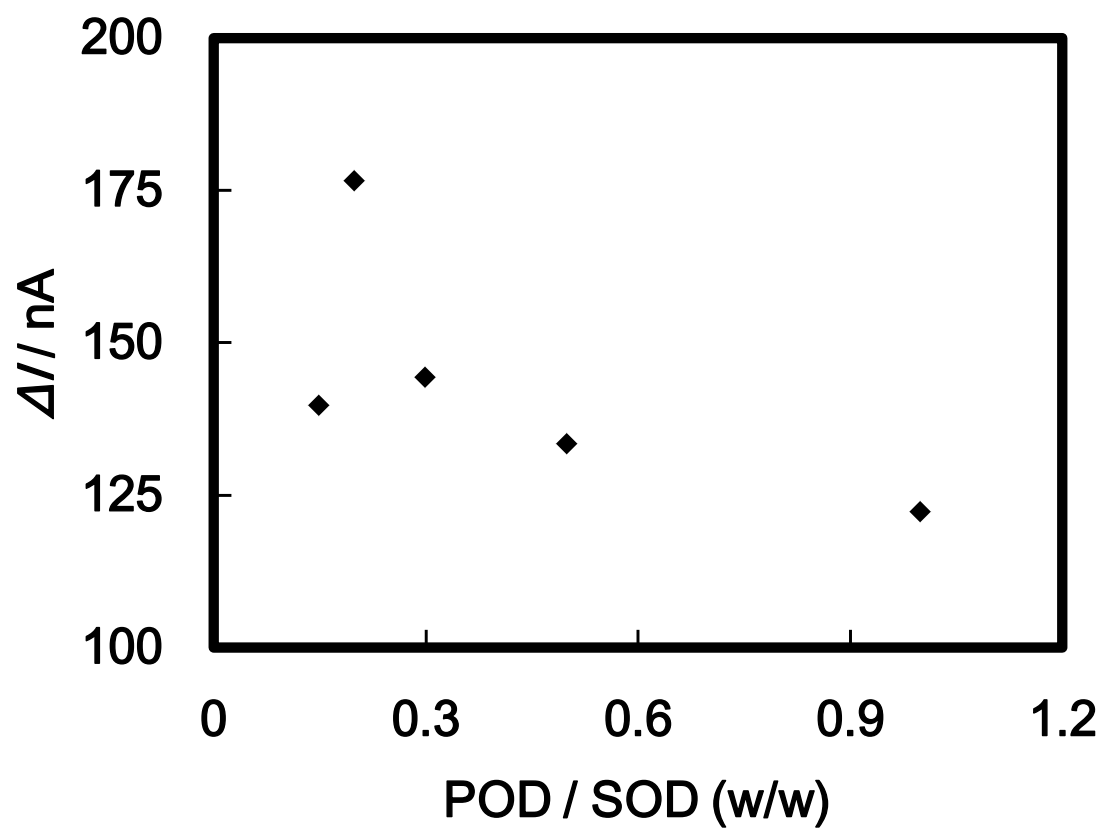
e$^-$

PVI[Fe(CN)$_5$]$^{2-}$

POD

H$_2$O$_2$

PVI[Fe(CN)$_5$]$^{3-}$

H$_2$O
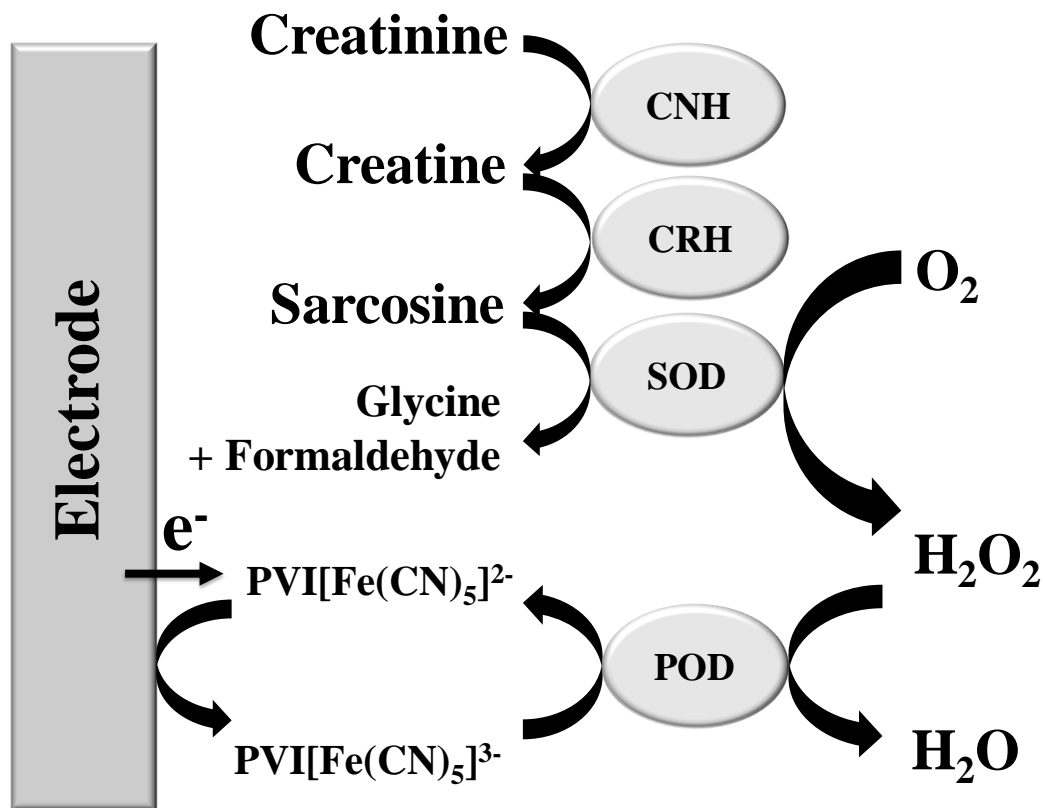
Scheme 1

Figure 1

Figure 2

Figure 3
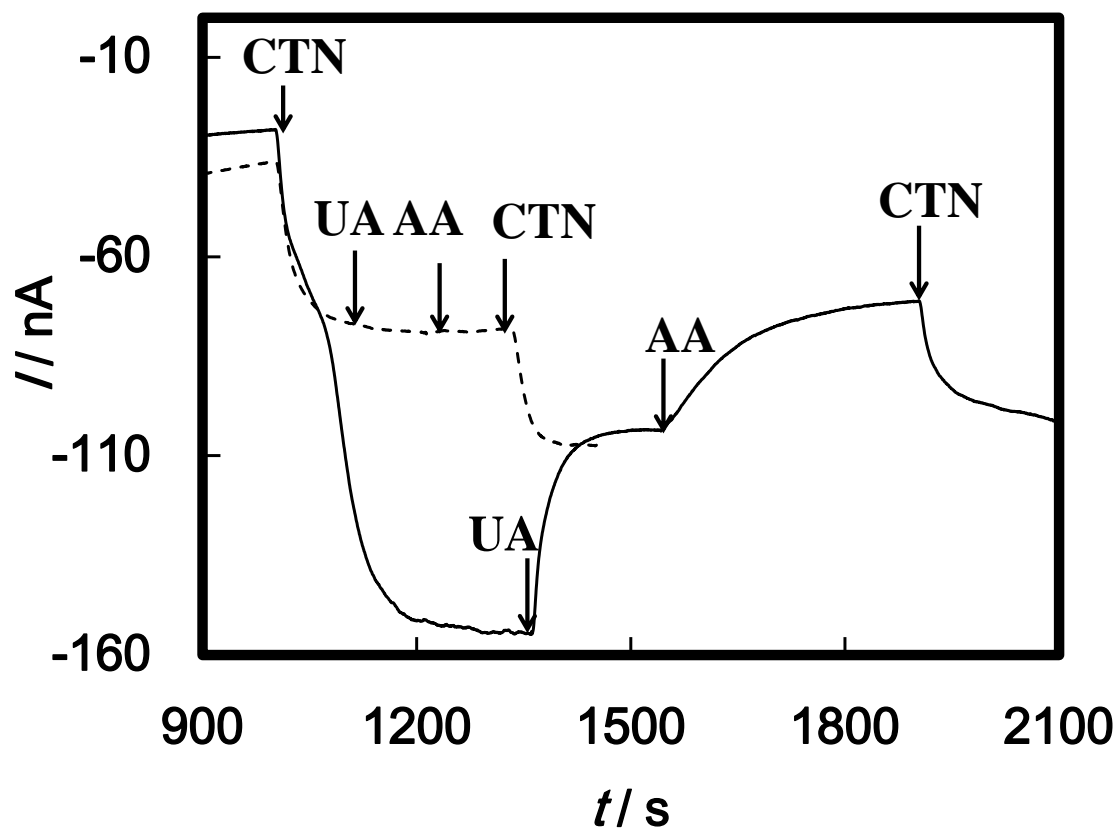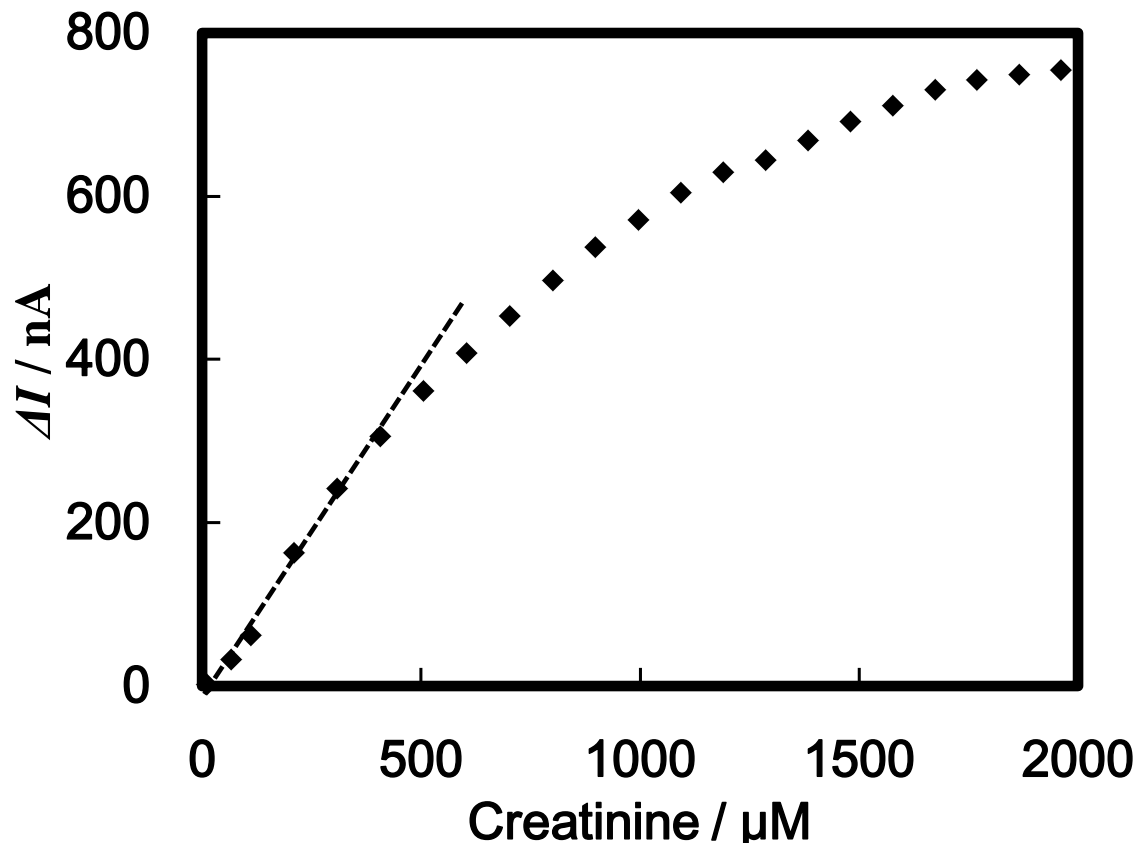
Figure 4

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.