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**Amperometric Biosensor Based on Reductive H₂O₂ Detection Using
Pentacyanoferrate-bound Polymer for Creatinine Determination**

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14 Abstract

15 Pentacyanoferrate-bound poly(1-vinylimidazole) (PVI[Fe(CN)₅]) was selected as a
16 mediator for amperometric creatinine determination based on the reductive H₂O₂
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 O₂. 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

- 32 Keywords: Pentacyanoferrate-bound poly(1-vinylimidazole); Creatinine
- 33 amidohydrolase; Reductive H₂O₂ detection; Peroxidase; Nafion; Urine creatinine test.

34 1. Introduction

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

50 Rather than CD, creatinine amidohydrolase (CNH), creatine amidohydrolase
51 (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:

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.

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) ($\text{PVI}[\text{Fe}(\text{CN})_5]$) is
85 selected as a mediator between POD and an electrode for creatinine determination
86 considering its poor mediating capability against SOD. $\text{PVI}[\text{Fe}(\text{CN})_5]$ 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 H₂O₂, then the reduction current of PVI[Fe(CN)₅] was observed by the H₂O₂ 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 ($\text{Na}_2[\text{Fe}(\text{CN})_5(\text{NO})]\cdot 2\text{H}_2\text{O}$), 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 $\text{PVI}[\text{Fe}(\text{CN})_5]$

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

117 PVI powder was obtained after filtering and drying. PVI[Fe(CN)₅] was then synthesized
118 as reported [19]. Briefly, 200 mg of Na₂[Fe(CN)₅(NO)]·2H₂O 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 ($0.5\text{--}2.5\text{ mg mL}^{-1}$)
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 $\lambda = 520\text{ nm}$ was measured with a spectrophotometer
145 (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)₂Cl] (PVI[Os(dmebpy)₂Cl]), 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)₂Cl] 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)₂Cl] electrode, the catalytic reduction current from POD reaction was hardly observed as shown in Fig. 1D.

164 The reason which causes the difference in the reactivity between PVI[Fe(CN)₅]
165 and PVI[Os(dmebpy)₂Cl] 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)₂Cl 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**

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 rigidity 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. $\text{Ag}|\text{AgCl}$) 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].

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[$\text{Fe}(\text{CN})_5$]-modified 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 H₂O₂ 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

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 $12\text{ }\mu\text{M}$ and the linear range is from 12 to $400\text{ }\mu\text{M}$ ($R^2 = 0.99$), 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, $10\text{ }\mu\text{L}$ 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 $\lambda = 520\text{ nm}$ 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

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.

261 **4. Conclusion**

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.

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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 $\text{PVI}[\text{Fe}(\text{CN})_5]$.

310

311 Figure 1

312 Cyclic voltammograms of (A) SOD- $\text{PVI}[\text{Fe}(\text{CN})_5]$ electrode, (B) SOD/POD-
313 $\text{PVI}[\text{Fe}(\text{CN})_5]$ electrode, (C) SOD- $\text{PVI}[\text{Os}(\text{dmebpy})_2\text{Cl}]$ electrode and (D) SOD/POD-
314 $\text{PVI}[\text{Os}(\text{dmebpy})_2\text{Cl}]$ 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 , $\text{PVI}[\text{Fe}(\text{CN})_5]$: 30 μg , $\text{PVI}[\text{Os}(\text{dmebpy})_2\text{Cl}]$: ca. 40
319 μg .

320

321 Figure 2

322 Dependence of the current response on the $\text{PVI}[\text{Fe}(\text{CN})_5]$ 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 -0.1 V.

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

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.