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Citation	Electrochemistry Communications (2010), 12(6): 839-842
Issue Date	2010-06
URL	<a href="http://hdl.handle.net/2433/128892">http://hdl.handle.net/2433/128892</a>
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Type	Journal Article
Textversion	author

1 **Bioelectrocatalytic endpoint assays based on steady-state diffusion current at**  
2 **microelectrode array**

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12

13 **Abstract**

14 Highly reproducible bioelectrocatalytic endpoint assays are described. The method is based on a  
15 complete redox conversion of a substrate to a redox mediator with a corresponding redox  
16 enzyme and an amperometric detection of the reduced mediator on a diffusionally independent  
17 microelectrode array. The current reaches a steady state within a few seconds and is proportional  
18 to the number of the integrated microelectrodes. The method has successfully been applied to  
19 histamine detection at micro-molar level and glucose detection at milli-molar level.

20

21 **Keywords:** Bioelectrocatalysis, Endpoint assay, Microelectrode array, Histamine assay, Glucose  
22 assay

23

## 24 **1. Introduction**

25

26 Electrochemical analysis with biological functions such as biosensors have been received  
27 much attention in the field of clinical analysis, food industry and environmental monitoring,  
28 hence numerous efforts have been devoted to develop rapid, reliable and accurate  
29 electrochemical biosensors [1]. However, most of the conventional amperometric biosensors  
30 have an intrinsic disadvantage in the issue that the response current is liable to be affected by  
31 either enzyme kinetic-related factor or diffusion-related factor of substrate (and mediator in  
32 mediated bioelectrocatalysis) [2]. This issue is frequently addressed by using permeable  
33 membrane-coated electrodes with raised enzyme activity under convectional conditions [3].  
34 However, it is difficult to strictly control the permeability of membranes on miniaturized  
35 biosensors and to realize high reproducibility. Coulometry as an absolute quantitative analysis is  
36 an alternative of bio-sensing [4, 5], but is often time-consuming.

37 Spherical diffusion plays an important role at microelectrode and provides steady-state  
38 current even under quiescent conditions [6]. In our best knowledge, however, there is no report  
39 on micro-biosensors utilizing such spherical diffusion. One of the reasons is that the current is  
40 usually within or below nano-ampere range, which might not be convenient for practical use.  
41 Microelectrode array (MEA) is designed by integration of several microelectrodes to increase  
42 the current intensity in various amperometric sensors [7–11]. In MEAs, the distance between  
43 microdisc electrodes should be larger than 12 times of the radius ( $r$ ) in order to ensure  
44 diffusional independence of the microelectrodes [12], although the geometry and the shape of  
45 microelectrodes are important factors determining the timescale [9–11]. It is not easy to  
46 fabricate such ideal MEAs, and diffusion-layer overlapping seems to occur at most of MEAs.  
47 The other reason is that sufficiently high enzyme activity is required to satisfy the  
48 diffusion-controlled condition of substrate in bioelectrocatalytic reactions.

49           Herein, we describe a reproducible amperometric detection method by monitoring  
50 steady-state diffusion current in mediated bioelectrocatalysis. First, we will propose a  
51 fabrication method of an diffusionally independent MEA. In addition, the endpoint method was  
52 incorporated in order to eliminate the effect of enzyme activity change and to increase the  
53 applicability of the method in practical bioassays, where the electron equivalent of substrate is  
54 completely transferred to that of mediator with the aid of redox enzyme in solution. The  
55 concentration of the reduced mediator (for the oxidation of substrate) is determined from the  
56 steady-state current at the MEA. The amperometric endpoint assays were applied to determining  
57 histamine at micro-molar level with histamine dehydrogenase (HmDH) and ferricyanide (as a  
58 mediator) and glucose at milli-molar level with pyrroloquinoline quinone (PQQ)-dependent  
59 glucose dehydrogenase (PQQ-GDH) and benzoquinone (as a mediator). The performance will  
60 be discussed in detail.

61

## 62 **2. Experimental**

63

### 64 *2.1. Reagents and materials*

65           Recombinant HmDH was expressed in *Escherichia coli* and purified as described  
66 previously [13]. The protein concentration was determined using a modified Lowry method  
67 with a DC Protein Assay Kit (Bio-Rad, USA) with bovine serum albumin as a standard protein.  
68 PQQ-GDH (EC 1.1.5.2 4200 U mg<sup>-1</sup>) was obtained from Amano Enzyme (Japan). Mutarotase  
69 (EC 5.1.3.3 913 U mL<sup>-1</sup>) was purchased from Oriental Yeast (Japan). All other chemicals were  
70 of analytical reagent grade and were used without further purification. D-Glucose stock solution  
71 was prepared with a phosphate buffer (0.1 M, pH 7) and stored overnight to reach the  
72 mutarotative equilibrium. Epoxy resin (Alardite 2020) and silver-epoxy resin (Dotite D-753)  
73 were obtained from Huntsman (USA) and Fujikura Kasei (Japan), respectively.

74

75 *2.2. Fabrication of microelectrode array (MEA)*

76 The fabrication scheme and the structure of our MEA are illustrated in Fig. 1. Firstly, a Pt  
77 wire (20  $\mu\text{m}$  in diameter; Niraco, Japan) was inserted into a silicon tube (i.d. 0.5 mm). The tube  
78 was filled half with the epoxy resin (overnight, 55  $^{\circ}\text{C}$ ). The silver-epoxy resin was crammed into  
79 the tube from the opposite side (4 h, 55  $^{\circ}\text{C}$ ). The resin was ejected from the silicon tube and is  
80 called Pt-embedded rod. A blank rod was also prepared in the same manner without using Pt  
81 wire. The Pt-embedded rods and the blank rods were inserted together in a silicon tube (i.d. 3  
82 mm) (Fig. 1a). The Pt-embedded rods were arrayed to keep away from the neighboring ones in a  
83 distance between the Pt wires of at least 500  $\mu\text{m}$  (Fig. 1b). The number of the Pt-embedded rod  
84 in the MEA ( $N$ ) was varied from 1 to 7. In this paper, a single microelectrode is described as  
85 MEA with  $N = 1$ , for simplification in description. After arraying the rods, the space among the  
86 rods was filled with the epoxy resin. The rod array was then ejected from the silicon tube and  
87 mounted in a glass tube (i.d. 3 mm). The silver-epoxy resin was crammed into the glass tube  
88 from the opposite side and a lead wire was inserted in the silver-epoxy resin for the electrical  
89 connect with the Pt-embedded rods. The glass tube was filled with the epoxy resin in the back  
90 side (Fig. 1c). The surface of the MEA was polished to a flat and mirror finish with sandpaper  
91 (#1500) and alumina slurry (0.05  $\mu\text{m}$ ).

92

93 *2.3. Electrochemical measurements and endpoint assays of histamine and glucose with MEA*

94 Cyclic voltammetry and amperometry were carried out in a three-electrode system on an  
95 ALS CHI 611B electrochemical analyzer (BAS Inc.) equipped with a faraday gage in a  
96 laboratory-made electrolysis cell at a total volume of 1.0 mL. The MEA, a Pt wire and an  
97 Ag|AgCl|KCl (sat.) were used as the working, counter and reference electrodes, respectively. All  
98 potentials in this paper are referred to the reference electrode. The total volume of the analytical

99 solution was 1.0 mL.

100 In histamine detection, 10  $\mu\text{L}$  of a histamine sample solution was added to 1.0 mL of 0.1  
101 M phosphate buffer (pH 7.0) containing 500 nM HmDH and 5 mM  $\text{K}_3[\text{Fe}(\text{CN})_6]$ . After  
102 incubation at room temperature for 20 min, the amperometric detection was performed at +0.6 V.  
103 In glucose detection, 100  $\mu\text{L}$  of a glucose sample solution was added to 1.0 mL of 30 mM  
104 MOPS buffer (pH 7.0) containing 21 U PQQ-GDH, 9 U mutarotase, 25 mM benzoquinone and  
105 3 mM  $\text{CaCl}_2$ . After incubation at room temperature for 15 min under anaerobic conditions with  
106 Ar, the amperometric detection was performed at +0.85 V under quiescent conditions.

107

### 108 **3. Results and discussion**

109

#### 110 *3.1. Characterization of the MEA*

111 All of the MEAs ( $N = 1, 3, 7$ ) showed typical sigmoidal steady-state shape for  $[\text{Fe}(\text{CN})_6]^{3-}$   
112 at 20  $\text{mV s}^{-1}$  and the current was proportional to  $N$  (Fig. 2). Such sigmoidal response was  
113 observed at scan rates of 5–100  $\text{mV s}^{-1}$  and was independent of the scan rate. These results  
114 support that the steady-state spherical diffusion layer of each microelectrode is not overlapped  
115 with that of the adjacent ones. The steady-state limiting current ( $i_{\text{lim}}$ ) is given by:

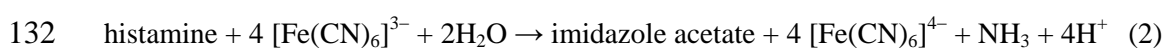
$$116 \quad i_{\text{lim}} = 4NnFrDc \quad (1)$$

117 where  $n$ ,  $D$ , and  $c$  are the number of electron, diffusion coefficient, and the bulk concentration  
118 of analyte, respectively.  $F$  is the Faraday constant. Thus, once  $NrD$  is determined experimentally  
119 with a given MEA,  $c$  can be easily evaluated from  $i_{\text{lim}}$ . In potential-step chronoamperometry, the  
120 current reached into almost steady state within 2 s, as shown in the inset of Fig. 2. In the  
121 following, the current after 5 s was taken as  $i_{\text{lim}}$ .

122

#### 123 *3.2. Responses to histamine at the MEA*

124 Histamine analysis is one of the major concerns in clinical and food chemistry because  
125 histamine is a transmitter in the nervous system and a powerful biological marker of food  
126 freshness [14, 15]. HmDH catalyzes a 2-electron oxidation of histamine to give imidazole  
127 acetaldehyde [16]. Several redox compounds such as  $[\text{Fe}(\text{CN})_6]^{3-}$  and osmium (III) complexes  
128 work as electron acceptors of the reduced HmDH [17, 18]. In addition, the product imidazole  
129 acetaldehyde is proposed to be oxidized non-enzymatically to imidazole acetate by some  
130 electron acceptor in solution [17]. When  $[\text{Fe}(\text{CN})_6]^{3-}$  was used as an electron acceptor, the  
131 overall reaction is expected as follows.



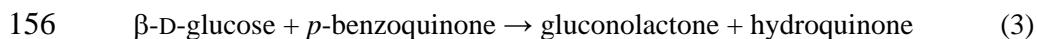
133 The succeeding non-enzymatic 2-electron oxidation will increase sensitivity twice in the  
134 histamine determination.

135 The  $i_{\text{lim}}$  of  $[\text{Fe}(\text{CN})_6]^{4-}$  in the endpoint assay detection with the MEA ( $N = 7$ ) was in a  
136 very nice linear correlation to  $c(\text{histamine})$  with a correlation coefficient larger than 0.999 over a  
137 wide range of  $c(\text{histamine})$  from 10 to 1000  $\mu\text{M}$ . The detection limit was 8.0  $\mu\text{M}$  at an  $S/N$  ratio  
138 of 3, which is higher than some reported works [17–20]. In addition, the detection limit can be  
139 improved by increasing the number of embedded microelectrodes [7]. The relative standard  
140 deviation was 4.1% for five separate measurements at 10  $\mu\text{M}$  histamine, in which the MEA  
141 surface was polished with alumina slurry before each measurement. The sensitivity ( $4nFrD$ )  
142 was  $6.6 \times 10^{-2} \text{ A M}^{-1}$  for  $N = 7$  and was proportional to  $N$  ( $2.9 \times 10^{-2} \text{ A M}^{-1}$  for  $N = 3$  and  $9.2 \times$   
143  $10^{-3} \text{ A M}^{-1}$  for  $N = 1$ ). From the result, the averaged  $4nFrD$  value is evaluated as  $9.5 \times 10^{-3} \text{ A}$   
144  $\text{M}^{-1}$  for the histamine analysis with the MEA. In a separate experiment, the  $4FrD$  value of  
145  $[\text{Fe}(\text{CN})_6]^{4-}$  was evaluated as  $2.4 \times 10^{-3} \text{ A M}^{-1}$  at the MEA in 0.1 M phosphate buffer (pH 7.0).  
146 The comparison of the two values can verify our expectation that  $n = 4$  (Eq. 2).

147

148 *3.3. Responses to glucose at the MEA*

149 Metabolic disorders like diabetes mellitus are reflected by human blood glucose  
150 concentrations higher or lower than the normal range of 4.4–6.6 mM [21]. When we apply our  
151 amperometric endpoint assay to such high concentrations of the analyte, very fast enzymatic  
152 conversion is required. PQQ-GDH catalyzes the 2-electron oxidation of  $\beta$ -D-glucose with  
153 several artificial electron acceptors. The activity is very high [22]. From the kinetic viewpoint,  
154 we selected *p*-benzoquinone as an electron acceptor in the PQQ-GDH reaction [23]. The overall  
155 reaction is written by:



157 We first tried to amperometric trace of the enzyme kinetics by measuring time  
158 dependence of  $i_{\text{lim}}$  of hydroquinone generated in the reaction of Eq. (3). The PQQ-GDH reaction  
159 appeared to reach an endpoint within 15 min under the present conditions, but  $i_{\text{lim}}$  was gradually  
160 increasing after 15 min. This is due to the mutarotation. Therefore, it is essential to accelerate  
161 the mutarotation proceeding simultaneously with the PQQ-GDH reaction. For this purpose,  
162 mutarotase was added in the enzyme solution. Under such conditions, the  $i_{\text{lim}}$  reached a constant  
163 value within 15 min. Furthermore, the steady-state value of  $i_{\text{lim}}$  is accounted for 161% of that in  
164 the absence of mutarotase (at 15 min). The value is very good agreement with the concentration  
165 ratio of the total glucose against  $\beta$ -D-glucose in mutarotative equilibrium. These results indicate  
166 that the complete redox conversion of the total glucose to hydroquinone is successfully achieved  
167 under the present conditions.

168 The  $i_{\text{lim}}$  for the glucose determination showed a linear relationship against  $c(\text{glucose})$   
169 with correlation coefficients larger than 0.998. The relative standard deviation for five  
170 independent measurements was 1.2% at  $c(\text{glucose}) = 5.0 \text{ mM}$  with the MEA ( $N = 7$ ).

171 The sensitivity ( $= 4NnFrD$ ) was  $4.0 \times 10^{-2} \text{ A M}^{-1}$  for  $N = 7$ ,  $1.8 \times 10^{-2} \text{ A M}^{-1}$  for  $N = 3$ ,  
172 and  $5.7 \times 10^{-3} \text{ A M}^{-1}$  for  $N = 1$ . By considering that  $n = 2$  in this case, the average value of  $4FrD$   
173 is evaluated as  $2.8 \times 10^{-3} \text{ A M}^{-1}$ . The value is in good agreement with that ( $2.8 \times 10^{-3} \text{ A M}^{-1}$ )



174 evaluated from separate experiments on  $i_{lim}$  of hydroquinone in the same buffer solution  
175 containing the enzymes.

176

#### 177 **4. Conclusion**

178 The diffusionally independent MEA electrode proposed here gives very stable and  
179 reproducible  $i_{lim}$ . Once the conditional parameter  $4NnFrD$  is estimated, the bulk concentration  
180 of analyte can be easily determined from  $i_{lim}$  within a few seconds. The amperometric method  
181 can be successfully combined with endpoint enzyme assays using mediated bioelectrocatalysis,  
182 in which  $i_{lim}$  of the mediator generated in the enzyme reaction was determined with the MEA.  
183 The principle is applicable to almost all redox enzymatic assays, including micro-plate analysis.  
184 Selection of better mediator will improve the proposed method into the practical use level.

185

#### 186 **References**

- 187 1. B.J. Privett, H.S. Jae, M.H. Schoenfish, *Anal. Chem.*, 80 (2008) 4499-4517.
- 188 2. B. Limoges, J. Moiroux, J.-M. Savéant, *J. Electroanal. Chem.* 521 (2002) 1-7.
- 189 3. W.J. Albery, P.N. Bartlett, *J. Electroanal. Chem.* 194 (1985) 211-222.
- 190 4. M. Fukaya, H. Ebisuya, K. Furukawa, S. Akita, Y. Kawamura, S. Uchiyama, *Anal. Chim.*  
191 *Acta* 306 (1995) 231-236.
- 192 5. S. Tsujimura, A. Nishina, Y. Kamitaka, K. Kano, *Anal. Chem.*, 81 (2009) 9383-9387.
- 193 6. K. Aoki, *Electroanalysis*, 5 (1993) 627-639.
- 194 7. O. Ordeig, J. del Campo, F.X. Muñoz, C.E. Banks, R.G. Compton, *Electroanalysis* 19  
195 (2007) 1973-1986.
- 196 8. X.-J. Huang, A.M. O'Mahony, R.G. Compton, *Small* 5 (2009) 776-788.
- 197 9. T.J. Davies, S. Ward-Jones, C.E. Banks, J. del Campo, R. Mas, F.X. Muñoz, R.G. Compton,  
198 *J. Electroanal. Chem.*, 585 (2005) 51-62.

- 199 10. T.J. Davies, R.G. Compton, *J. Electroanal. Chem.*, 585 (2005) 63-82.
- 200 11. D. Menshykau, X.J. Huang, N.V. Rees, F.J. del Compo, F.X. Muñoz, R.G. Compton,  
201 *Analyst*, 134 (2009), 343-348.
- 202 12. Y. Saito, *Rev. Polarogr.* 15 (1968) 177-186.
- 203 13. N. Fujieda, N. Tsuse, A. Satoh, T. Ikeda, K. Kano, *Biosci. Biotechnol. Biochem.* 69 (2005)  
204 2459-2462.
- 205 14. M.A. Beaven, A. Robinson-White, N.B. Roderick, G.L. Kauffman, *Klin. Wochenschr.* 60  
206 (1982) 873-881.
- 207 15. L. Lehane, J. Olley, *Int. J. Food Microbiol.* 58 (2000) 1-37.
- 208 16. M. Tsutsumi, N. Fujieda, S. Tsujimura, O. Shirai, K. Kano, *Biosci. Biotechnol. Biochem.*  
209 72 (2008) 786-796.
- 210 17. R. Yamada, N. Fujieda, M. Tsutsumi, S. Tsujimura, O. Shirai, K. Kano, *Electrochemistry*  
211 76 (2008) 600-602.
- 212 18. K. Takagi, S. Shikata, *Anal. Chim. Acta* 505 (2004) 189-193.
- 213 19. M. Niculescu, I. Frébort, P. Peč, P. Galuszka, B. Mattiasson, E. Csöregi, *Electroanalysis* 12  
214 (2000) 369-375.
- 215 20. M.G. Loughran, J.M. Hall, A.P.F. Turner, V.L. Davidson, *Biosens. Bioelectron.* 10 (1995)  
216 569-576.
- 217 21. J. Wang, *Chem. Rev.* 108 (2008) 814-825.
- 218 22. L. Ye, M. Hämmerle, A.J.J. Olsthoorn, W. Schuhmann, H.-L. Schmidt, J.A. Duine, A.  
219 Heller, *Anal. Chem.*, 65 (1993) 238-241.
- 220 23. N. Okumura, T. Abo, S. Tsujimura, K. Kano, *Electrochemistry* 74 (2006) 639-641.
- 221

222 **Figure Captions**

223 Fig. 1. (a) Schematic diagram of the integration process, (b) the photo image and (c) the

224 structure of MEA.

225

226 Fig. 2. Cyclic voltammograms obtained at MEA with  $N =$  (a) 1, (b) 3 and (c) 7 for 1 mM

227  $[\text{Fe}(\text{CN})_6]^{3-}$  in 1 M KCl, at a scan rate  $20 \text{ mV s}^{-1}$ . Inset: chronoamperometric responses for

228  $[\text{Fe}(\text{CN})_6]^{4-}$  in 1 M KCl in potential step from  $-0.1 \text{ V}$  to  $+0.6 \text{ V}$  at MEA ( $N = 1, 3, 7$  from

229 bottom to top).

