The Effects of Chain Length of Ferrocene Moiety on Electrical Communication of Mediators- and Enzymes-modified Electrodes

Yoshitomo KASHIWAGI, Qinghai PAN, Yoshinori YANAGISAWA,

Norihiko SIBAYAMA and Tetsuo OSA*

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Electrical communication between alcohol dehydrogenase and graphite electrode is established in a domain of poly(acrylic acid) thin layer coated on graphite felt in which ferrocene, NADH, diaphorase and alcohol dehydrogenase are chemically immobilized. It was confirmed that NADH was not directly oxidized with ferrocene derivatives and needed the intervention of diaphorase between ferrocene derivatives and NADH. The effects of ferrocene derivatives with different alkyl chain lengths ($\text{Fc-CH}_2\text{NH}(\text{CH}_2)_n\text{NH}_2$, $n=4\sim 12$) to electromediatory oxidations of NADH and alcohol dehydrogenase were examined and the best result was obtained by the use of the flexible ferrocene derivatives with spacer length of $12\sim 15\text{ \AA}$ ($n=6\sim 8$).

1 INTRODUCTION

We have recently succeeded in electro-enzymatic oxidation of cyclohexanol on a thin poly(acrylic acid) (PAA) layer coated graphite felt (GF) electrode in which layer all mediators and enzymes were chemically immobilized¹⁾. To attain electroenzymatic oxidation of alcohols with alcohol dehydrogenase (ADH), electron-transfer mediators between electrode and ADH are indispensable. If ferrocene (Fc) is used as a mediator, redox enzyme such as diaphorase (Dp) and coenzyme of reduced nicotinamide adenine dinucleotide (NADH) must be intervened to communicate the electron transfers between Fc and ADH. Therefore, immobilization of four components such as Fc, Dp, NADH and ADH to the electrode surface and smooth electron transfer among them are necessary to construct a perfect "bioelectrochemical reactor", that is, the complete absence of the essential components for electro-enzymatic oxidation in electrolyte solution.

Before the present study, we succeeded in electroenzymatic oxidation of several kinds

of alcohols on a thin PAA layer coated GF electrode immobilizing ferrocenylethylamine, Dp and ADH in the presence of NADH in phosphate buffer (pH 7.2)²⁾. Then, we have examined further immobilization of NADH on the electrode. To attain a smooth electrical communication among four essential components in a domain of PAA layer which is formed by crosslinking with hexamethylenediamine, we have adopted the immobilization of a different arm length of Fc derivatives, as the most convenient way, to be a flexible and shuttle Fc moiety. This strategy will be also useful to exclude the problem of the complicated electron transfer process of the system, because the chemical treatments of enzymes must be minimum to suppress their deactivation.

We, at first, report the cyclic voltammetric results and immobilization amounts of Fc derivatives with 5~15 bond chain attached to the PAA layer on GF electrode by amido bond, then, similar results of NADH immobilization, and finally the electrical communication on the system of all essential components-modified electrodes, though the same modified amounts of each component were not attained due to using the same reaction conditions.

Pharmaceutical Institute, Tohoku University (Aobayama, Sendai 980-77 Japan)

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2 EXPERIMENTAL

2.1 Reagents

GF (National Electric Carbon Corp., WDF) was kindly gifted by Prof. J. M. Bobbitt, Univ. of Connecticut. Dp (E.C. 1.8.1.4) and ADH (E.C. 1.1.1.1) were purchased from Behliger, and NADH from ORIENTAL YEAST. Dry PAA (av. MW of 1,400,000) were prepared by freeze-drying a 25% aqueous solution obtained from Wako Pure Chemical Industries. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (Dojindo Laboratories, WSC), ferrocenecarboxaldehyde (Aldrich Chemical Company), alkylenediamines ($n=2, 4, 6, 8, 10$ and 12 , Tokyo Kasei Co.), and sodium borohydride (NaBH_4 , Nacalai Tesque Inc.) were used as they stand. N,N -Dimethylformamide (DMF, guaranteed reagent), dichloromethane (CH_2Cl_2) and methanol (CH_3OH) were used without any further purifications.

2.2 Synthesis of ferrocene derivatives

Six kinds of ferrocenylaminoalkylamine ($\text{Fc-CH}_2\text{NH}(\text{CH}_2)_n\text{NH}_2$, $n=2, 4, 6, 8, 10$ and 12) were synthesized according to established procedures³⁾. A 4-fold excess of the appropriate diamine was heated in 100 cm^3 of DMF to 100°C , and 500 mg of ferrocenecarboxaldehyde dissolved in 50 cm^3 of DMF was added dropwise within 1 h to prevent formation of the bridged diferrocene byproducts. After another hour an excess of NaBH_4 in water was dropped into the solution, and the reaction mixture was stirred for an additional hour at room temperature. The solution was changed to pH 3 with 2 M ($\text{M}=\text{mol}\cdot\text{dm}^{-3}$) HCl , and was stirred for 24 h at room temperature (NaBH_4 was decomposed). The solution was changed to pH 9 with 2 M NaOH (make the same pH as before). The solvent mixture was rotavaporated to dryness and the residue extracted with CH_2Cl_2 and separated through a silica column ($1.5\text{ cm}\times 30\text{ cm}$). A first fraction (the bridged diferrocene) was eluted with CH_2Cl_2 , the main fraction with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ 1:1. The solvent was evaporated to dryness. The yields of ferrocenylaminoalkylamine ($n=2, 4, 6, 8, 10$ and 12) were 48, 41, 39, 27, 26 and 14 %, respectively. All compounds showed the expected ^1H NMR spectra.

2.3 Preparation of mediators-modified GF

electrodes

GF electrode ($5.0\times 2.0\times 0.5\text{ cm}^3$) was coated with PAA by immersion in a 0.25 % methanol solution and dried in air. The PAA film-coated GF electrode (ca. 40 nm thick PAA) was attached with ferrocenylaminoalkylamine, crosslinked with hexamethylenediamine successively^{2,4)}.

The modified GF electrode thus prepared was reacted with 5 cm^3 of 60 mM NADH in phosphate buffer (pH 7.2) in the presence of WSC (10 equiv. to NADH) at 4°C for 72 h , dried in *vacuo*, washed in 50 cm^3 water for three times and dried in *vacuo*. Then the electrode was treated with 5 cm^3 of $50\text{ }\mu\text{M}$ Dp in phosphate buffer (pH 7.0) in the presence of WSC (10 equiv. to Dp) at 4°C for 72 h , washed in 50 cm^3 water for three times and dried in *vacuo*⁴⁾. Then the electrode was treated with 5 cm^3 of $50\text{ }\mu\text{M}$ ADH in phosphate buffer (pH 7.0) in the presence of WSC (10 equiv. to Dp) at 4°C for 72 h , washed in 50 cm^3 water for three times and dried in *vacuo*.³⁾

2.4 Electrochemical measurements

Cyclic voltammetry was carried out in phosphate buffer (pH 7.0). Mediators-modified GF electrode ($1.0\times 1.0\times 0.5\text{ cm}^3$) was employed as a working electrode, and a platinum wire was as a counter electrode. The anode potentials were referred to SCE. Electrode potential was controlled with a potentiostat/function generator (CS-1090, Cypress System, Inc., USA). All electrochemical measurements were carried out at room temperature ($20\pm 2^\circ\text{C}$).

3 RESULTS AND DISCUSSION

3.1 Cyclic voltammetry on GF electrodes modifying Fc derivatives with different alkyl chain lengths

To attain an effective electron transfer between electrode and Fc moiety modified on the electrode, Fc moiety will be necessary to possess some lengths of spacer chain so that Fc moiety can shuttle flexibly near electrode surface. For the purpose, ferrocenylaminoalkylamines attaching the different alkyl chain lengths of $n=2, 4, 6, 8, 10$, and 12 in the structure of $\text{Fc-CH}_2\text{NH}(\text{CH}_2)_n\text{NH}_2$ were immobilized to the carboxylic acid groups of the PAA layer coated on GF by amido bond under the same

Fig. 1 Cyclic voltammograms on different chain lengths of Fc-modified GF electrode ($1.0 \times 1.0 \times 0.5 \text{ cm}^3$) (—) and different chain lengths of Fc-, NADH-, Dp- and ADH-multimodified GF electrode ($1.0 \times 1.0 \times 0.5 \text{ cm}^3$) (---) in phosphate buffer (pH 7.0) at the scan rate of 2 mV/s.

carbodiimide (DCC). The cyclic voltammograms (CVgrams) of the Fc derivatives-modified electrodes ($1.0 \times 1.0 \times 0.5 \text{ cm}^3$) in phosphate buffer (pH 7.0) are shown in Fig. 1. Using this figure, the values of the oxidation peak current (i_{pa}) of each Fc derivatives-modified electrode were plotted toward the alkyl chain length as shown in Fig. 2A. The order of i_{pa} values was $n=8(14.6 \text{ \AA}) \approx 6(12.1 \text{ \AA}) \approx 4(9.6 \text{ \AA}) \approx 2(7.0 \text{ \AA}) > 10(17.1 \text{ \AA}) > 12(19.6 \text{ \AA})$. The spacer bond length between the carbon of Fc and the carbon of PAA corresponds to $1.26n + 5.8 \text{ \AA}$. The values to each n are shown in the parentheses followed by the numerals of n . The half wave potential ($E_{1/2}$) values and the difference values between the oxidation potential (E_{ox}) and the reduction potential (E_{red}) of Fc moieties on Fc derivatives-modified electrodes were also plotted against the alkyl chain length as shown in Fig. 2B and 2C, respectively. The $E_{1/2}$ values in Fc moieties of $n=6$ and 8 were more positive than those of $n=2, 4, 10$ and 12, and the difference values between $E_{ox}-E_{red}$ were small in Fc moieties of $n=2$

Fig. 2 Peak current (i_{pa} , A), half-wave potential ($E_{1/2}$, B) and difference between oxidation potential and reduction potential ($E_{ox} - E_{red}$, C) of Fc-modified GF electrodes ($1.0 \times 1.0 \times 0.5 \text{ cm}^3$) against different alkyl chain length (n).

and 4 (80-95 mV) and large in those of $n=6, 8, 10$ and 12 (120-135 mV). These results suggest that the Fc derivatives-modified electrode of $n=2$ and 4 are better than those of $n=6$ and 8 on the viewpoint of electron transfer between GF electrode and Fc moiety but the latter electrodes will be stronger as oxidative mediator than the former electrodes. The Fc derivatives-modified electrodes of $n=10$ and 12 were less suitable as mediator for electron transfer and oxidizability. Heller et al. also reported that Fc-modified glucose oxidase with different spacer lengths revealed different electro-enzymatic ability of glucose oxidase between the electrode and Fc moiety and the communication was effective when the chains were long (> 10 bonds, i.e. $> n=7$) but not when the chains were short (< 5 bonds, i.e. $< n=2$)³.

3.2 The immobilized amounts of Fc derivatives with different alkyl chain

lengths to PAA layer coated on GF electrodes

The immobilized amounts by percent of Fc moiety to the carboxylic acid groups of the PAA layer calculated by means of the areas of CVgrams together with the percents obtained by the increase in weight and decrease in the remained carboxylic acid groups by back titration are shown in Table 1. The percent values by weight and titration

Table 1 Percents of immobilized Fc and NADH to the COOH groups of PAA layer coated on GF.

Number of alkyl chain of ferrocenylaminoalkylamine (n)	2	4	6	8	10	12
Spacer bond length / Å	7.0	9.6	12.1	14.6	17.1	19.6
Immobilized Fc to PAA						
by weight	33	29	41	41	33	27
by titration	39	32	43	45	33	30
by CVgram	4.0	3.5	4.5	4.5	3.0	1.6
Immobilized NADH to PAA						
measured by UV	11.1	13.7	12.7	13.6	8.1	9.4

were almost same and the order of the values was also same as the one indicated by i_{pa} against n except $n=4$. The results mean that the flexible Fc moiety of $n = 6$ and 8 gave the large immobilization values and longer chain length of Fc moiety of $n = 10$ and 12 gave small immobilization values probably due to a low reaction probability. The low immobilization of Fc moiety of $n = 2$ and 4 is no clear. The values obtained by CVgrams were about one tenth-fold obtained by weight and titration. The results suggest that only 10% of Fc moieties attached to the carboxylic acid groups of the PAA layer act as an electron-transfer mediator. In the TEMPO(2,2,6,6-tetramethylpiperidinyl-N-oxyl)-modified GF electrodes, the values obtained by weight, titration and CVgrams were 64, 64 and 51%, respectively, so that 81% of TEMPO moieties attached to the carboxylic acid groups of the PAA layer acted as mediator⁵⁾. Therefore, the ability of electron-transfer mediation has a big difference by the kind of mediator attached to the electrode.

3.3 The immobilized amounts of NADH on different spacer lengths of Fc derivatives- and NADH-modified GF electrodes

In order to calculate the amount of NADH immobilized to the carboxylic acid groups

of the PAA layer, Fc- and NADH-coimmobilized electrodes were dipped into methanol to be dissolved completely until NADH has not remained on the GF. Then, the concentration of NADH in methanol was measured by UV absorption at 340 nm. The values of NADH by percent were demonstrated in Table 1. Thirteen to fourteen percent of the carboxylic acid groups in the PAA layer were immobilized with NADH on Fc derivatives-modified electrodes of $n = 4, 6$ and 8 . The modified amounts of NADH were smaller for the Fc derivatives-modified electrode of $n = 2, 10$ and 12 than those of $n = 4, 6$ and 8 . The smaller amount in $n = 2$ may be ascribed to a steric hindrance of the rigid Fc moiety and those in $n = 10$ and 12 should be derived from suppression of NADH introduction to the carboxylic acid groups of the domain due to a flexible long chain of the Fc moieties. This immobilization is expected to proceed by the reaction of 6-amino group of adenine moiety of NADH with carboxylic acid groups of the PAA layer to form amido bond and was confirmed by the disappearance of primary amine absorption (1655 cm^{-1}) and appearance of amide II (1570 cm^{-1}) in IR spectra.

In the preparation of materials for affinity chromatography, redox-active species of NAD^+/NADH were bound to supports with long and flexible chain⁶⁾, where N^6 -position of adenine was reacted with sepharose derivatives by the formation of amino bonds. So far, however, no formation of amido bond between 6-amino-position of adenine and reagents was reported.

The immobilized amounts of NADH obtained by the weight and titration methods coincided with the amounts obtained by the UV method as discussed later (Table 3.).

3.4 Electromediation current in cyclic voltammograms on Fc- and NADH-co-modified electrode

The CVgrams of ferrocenylaminobutylamine ($n=4$)- and NADH-co-modified GF electrode in the presence and absence of Dp in phosphate buffer (pH 7.0) are shown in

Fig. 3 Cyclic voltammograms of Fc(n=4)-modified GF electrode (—), and Fc(n=4)- and NADH-comodified GF electrode in the absence (- - -) and the presence (— · —) of Dp in phosphate buffer (pH 7.0) at the scan rate of 2 mV/s. The concentration of Dp is 20 μ M; the electrode used is 1.0 x 1.0 x 0.5 cm^3 .

Fig. 3. The electrode measured in the absence of Dp indicated two oxidation peaks at 0.40 and 0.55 V in CVgrams which correspond to the separate oxidations of Fc and NADH, respectively. No electromediation current was observed. On the other hand, the electrode measured in the presence of Dp gave the mediation current at E_{pa} of the Fc moiety. This fact suggests that a direct electron-transfer between Fc and NADH is not possible and needs another mediator of Dp as expressed in eqs. (2), (3) and (4):

where the dash of Fc- and NADH- means that Fc and NADH are immobilized on the

Fig. 4 Cyclic voltammograms of Fc(n=4)-, NADH- and Dp-terminmodified GF electrode (- - -) and Fc(n=4)- modified GF electrode (—) in phosphate buffer (pH 7.0) at the scan rate of 2 mV/s. The electrode used is 1.0 x 1.0 x 0.5 cm^3 .

GF electrode. The Fc (n=4)-, NADH- and Dp-terminmodified electrode indicated the same electromediation curve (Fig. 4) as that on the Fc- and NADH-comodified electrode in the presence of Dp. The results mean that the Dp moiety attached to the PAA layer acts as electron-transfer mediator as reexpressed in eq. (5) instead of eq. (3).

The other chain lengths of Fc moiety of n = 6 and 8 gave the similar results as that of n = 4.

3.5 Electromediation current in cyclic voltammograms on Fc-, NADH-, Dp- and ADH-multimodified GF electrodes

In addition to immobilization of Fc, NADH and Dp to the PAA layer of the GF electrode, ADH was finally immobilized to the PAA layer. The CVgrams of the modified electrodes attaching different spacer lengths of ferrocenylaminoalkylamine (n=2~12) are shown in Fig. 1. All the CVgrams indicated electromediation curves, though the mediation current (i_{med}) was not increased by the immobilization of ADH. This result may be ascribed to a low immobilization of NADH as will be discussed in 3.6. However, the electrodes immobilizing all the components can oxidize cyclohexanol to cyclohexanone in high selectivity⁷⁾. Therefore, ADH immobilized to the PAA layer on GF electrodes is considered to be oxidized to oxidized ADH as expressed in eq. (6).

The values of i_{med} and the ratio of i_{med} over the peak current of Fc moiety itself, i_{med}/i_{pa} , on the GF electrodes attaching Fc derivatives with the different alkyl chain lengths are listed in Table 2. A high electromediation current and high ratio of i_{med}/i_{pa} were obtained on Fc (n=6 and 8)-, NADH-, Dp- and ADH-multimodified electrodes.

Table 2 Electromediation current (i_{med}) of Fc-, NADH-, Dp- and ADH-multimodified GF electrode and the ratio of i_{med}/i_{pa} by the different alkyl chain lengths of Fc to the PAA layer.

n	2	4	6	8	10	12
$i_{med}/\mu\text{A}$	370	380	480	530	290	200
i_{med}/i_{pa}	1.27	1.26	1.60	1.65	1.61	1.17

Therefore, it is concluded that the Fc ($n=6$ and 8)-, NADH-, Dp- and ADH-multi-modified electrodes are most suitable to the overall electron transfer from the electrode surface to ADH, though the Fc ($n=2$ and 4)-modified electrodes are better for the electron transfer from the electrode surface to the Fc moiety.

3.6 Determination of each modified amount of mediators and enzymes on GF electrodes

The amount of PAA coated on GF and the amount of Fc immobilized to the PAA layer were determined precisely using the increase of weight and the decrease of carboxylic acid groups in the PAA layer by titration. The amount of NADH immobilized to the PAA layer was obtained correctly using the UV method. We are temporarily determining the amounts of Dp and ADH by increase of weight and the decrease of the carboxylic acid groups in the PAA layer by titration. The percents of the reacted carboxylic acid groups for each compounds (in this case, Fc derivative of $n=6$ was used) obtained by weight and titration methods are summarized in Table 3. The immobilized amounts of

Table 3 Percents of reacted carboxylic acid groups of PAA layer for immobilization of Fc($n=6$)-, NADH-, Dp- and ADH GF measured by weight and titration methods.

measurement	Ratio of COOH groups of PAA (%)				
	Fc-modified	Cross-linked	NADH-modified	Dp-modified	ADH-modified
weight	41	11	11.5	0.14	0.39
titration	43	14	11.7	1.39	3.74

NADH obtained by the weight and titration methods agreed with each other and were same as the amount obtained by the UV method. The values of the bulky molecules of Dp and ADH immobilized to the PAA layer gave larger in the titration method than in the weight method. The results may be reasonable because a molecule of the enzymes has several reaction sites with carboxylic acid groups of the PAA. However, the values obtained by the weight method may include some amounts of adsorbed enzyme in the domain of the PAA layer, even if the modified GF was washed repeatedly. Therefore, the values of Dp and

ADH immobilized may not be correct and it is necessary to establish a correct method. Furthermore, more immobilization of the enzyme will be necessary for better electrical communication between the GF electrode and ADH intervened by Fc, Dp and NADH.

4 CONCLUSION

Comparing the spacer length (7~20 Å) of Fc derivatives attached to the carboxylic acid groups in the PAA layer coated on GF, Fc derivatives with spacer length of 7~10 Å ($n=2\sim4$) were more suitable for electrical communication and those with 12~15 Å ($n=6\sim8$) had stronger oxidizability, though only ca. 10% of Fc moiety acted as electron-transfer mediator.

The immobilized amounts of NADH were obtained correctly by each of UV, weight and titration methods. However, the immobilized amounts of Dp and ADH were small and necessary to improve the immobilizing method as well as establish a correct measurement method.

It was confirmed that NADH was not directly oxidized with Fc derivatives and needed the intervention of Dp between Fc derivatives and NADH. The Fc-, NADH-, Dp- and ADH-multimodified GF electrodes revealed the electromediatory oxidation of ADH immobilized on GF at the oxidation potential of Fc moiety. The spacer length of 12~15 Å ($n=6\sim8$) of Fc moiety was more effective for the electroenzymatic reaction. Thus, a complete electroenzymatic reactor in which all the mediators were immobilized on the electrode was constructed.

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