Electrochemical Characterization of Enzyme Electrodes Mediated by Ferrocene-Containing Acrylamide-Acrylic Acid Copolymers

Kenji YOKOYAMA*, Tomonori SHIBASAKI and Yuji MURAKAMI

Received May 17, 1996 ; Accepted July 16, 1996

Electron transfer between a ferrocene-based redox polymer and active site of glucose oxidase (GOD) is demonstrated. Crosslinked copolymers of acrylamide and acrylic acid with 11 bonds side chains containing ferrocene at the ends were synthesized. GOD and this redox polymer were co-reticulated on a glassy carbon electrode surface using glutaraldehyde and bovine serum albumin (BSA). BSA-based immobilization was highly effective to stabilize electrochemical response of the redox polymer. Four copolymers with different ferrocene contents were prepared, and electron transfer process of each polymer was explored. The $D_R^{1/2}C_R^*$ values were estimated to be around $10^{-9} \text{ mol cm}^{-2} \text{ s}^{-1/2}$. Electrochemical behavior of the redox polymer was found to be almost based on semi-infinite diffusion process. Thin hydrogel showed intermediate behavior between surface redox and diffusion-controlled processes. Catalytic electrochemistry of the enzyme electrodes was also investigated. The enzyme electrode with high ferrocene content revealed high catalytic current and low catalytic efficiency, $i_{\text{cat}}/i_{\text{redox}}$.

1 INTRODUCTION

Recently biosensors based on reagentless amperometric enzyme electrodes have been proposed¹⁻⁹⁾. They can be made by incorporating redox enzymes with a redox polymer that electrically connects the enzyme to an electrode. These devices rely on the electrical connection between enzyme redox centers and the electrode through the participation of redox centers in polymers. Long-range electron transfer to active sites of redox enzymes has been demonstrated recently, with enzymes both in solution and co-immobilized in two- and three- dimensional polymer structures on electrodes. The electrode sensitivity is dependent not only on the enzyme kinetics but on charge propagation through the film. This is particularly true when the enzyme

reaction is fast and/or the charge propagation through the polymer is slow. Thus, it is of importance for the understanding of the enzyme electrode dynamics to study electron transfer in the redox polymer used.

The polymer-modified electrodes that have been used include hydrogel polymers of poly(vinylpyridine)¹⁾, poly(1-vinylimidazole)^{2,3)}, poly(allylamine)⁴⁾ and poly(acrylamide)^{5,6)} modified by attachment of redox groups. Heller's group has reported crosslinked redox gels containing glucose oxidase (GOD) based on poly(vinylpyridine) with complexed Os(bpy)₂Cl (bpy = 2,2'-bipyridine)¹⁾. Free pyridine groups were quaternized to promote crosslinking with enzyme lysines. They have recently reported two new osmium-based redox hydrogels. These were formed by crosslinking poly(1-vinylimidazole) complexed with Os(bpy)₂Cl²⁾ or Os(4,4'-dimethyl-bpy)₂Cl³⁾ and poly(ethyleneglycol) diglycidyl ether in the presence of enzyme. Calvo and co-workers have reported ferrocene-containing acrylamide-acrylic acid copolymer for enzyme

School of Materials Science, Japan Advanced Institute of Science and Technology, (Tatsunokuchi, Ishikawa 923-12, Japan)

Key Words; Redox polymer, Glucose biosensor, Glucose oxidase.

electrode use⁵). Carboxylic group of the copolymer was covalently attached with aminoethylaminomethylferrocene, and GOD was immobilized with amide group via glutaraldehyde. However, due to the insufficient linkage between amide and aldehyde groups, the enzyme membrane may gradually be dissolved. Bu and co-workers have reported redox copolymer of acrylamide and vinylferrocene crosslinked with *N,N'*-methylenebisacrylamide (BIS), and GOD was simultaneously entrapped in the hydrogel at polymerization process⁶). However, this redox hydrogel required dialysis membrane to be fixed on the electrode.

A redox polymer that mediates electron transfer between enzymes and an electrode requires appropriate hydrophilicity, and hence a cross-linked polymer that forms hydrogel is suitable. However, a suitable technique for immobilization of the polymer and enzyme on an electrode should be developed.

In the present work we synthesized crosslinked copolymers of acrylamide and acrylic acid with flexible and long side chains containing ferrocene at the ends. Polyacrylamide is the most commonly used hydrogels for enzyme immobilization and electrophoresis because of its good chemical and mechanical stability and its inertness to microbial degradation. Rapid electron transfers between ferrocenes as well as between GOD and ferrocene can be realized using a polymer with a flexible and long side chain containing ferrocene. A schematic description of a polymeric unit is illustrated in Fig. 1. GOD and this redox hydrogel were immobilized on the electrode surface using glutaraldehyde. Electrochemical characteristics of the enzyme electrode fabricated were studied. Effect of bovine serum albumin (BSA) addition on stability was also investigated. Four copolymers with different ferrocene content were prepared, and electron transfer process and catalytic behavior of each polymer were explored.

2 EXPERIMENTAL

2.1 Chemicals

GOD (Sigma, Type-XS, from *Aspergillus niger*) was used. Acrylamide, acrylic acid, BIS, sodium borohydride, *N*-cyclohexyl-*N'*-(2-mor-

pholinoethyl) carbodiimide metho-*p*-toluenesulfonate, 1,6-diaminohexane and BSA were purchased from Wako. Ferrocene carboxaldehyde was from Aldrich, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate were from Eastman Kodak.

Fig. 1 Ferrocene-containing acrylamide-acrylic acid copolymer crosslinked with BIS.

2.2 Synthesis of redox polymer

Poly(acrylamide-acrylic acid) crosslinked with BIS was synthesized as described previously¹⁰). BIS (0.8 mmol) and the mixture (66 mmol) of acrylamide and acrylic acid were dissolved in a deoxygenated Tris-HCl buffer (24 mL). Four copolymers with different molar ratios of acrylamide to acrylic acid were synthesized and the feed ratios were 4:1, 1:1, 3:7 and 1:9. After addition of 0.5 mL of TEMED and 0.5 mL of ammonium persulfate solution (0.5 g/mL), the solution was placed under slight vacuum for about one hour. The gel block was pressed through 30 μ m-sieve. The granules were then thoroughly washed with water to remove non-polymerized monomers and the catalyst system.

The ferrocene derivative, aminoethylaminomethylferrocene, was prepared as reported previously¹¹). A 4-fold excess of the appropriate 1,6-diaminohexane was heated in 100 mL of dimethylformamide (DMF) to 100 °C, and 2.3 mmol of ferrocene carboxaldehyde dissolved in 50 mL of DMF was added dropwise within one hour

Table 1 XPS analysis of redox polymers synthesized.

	Feed ratio acrylamide / acrylic acid	Fe	N	O	C	(%)
RP-1	4 : 1	ND	ND	ND	ND	
RP-2	1 : 1	0.98	9.54	14.55	74.94	
RP-3	3 : 7	1.71	7.78	10.26	79.74	
RP-4	1 : 9	1.53	8.51	9.67	80.08	

ND: Not determined.

to prevent formation of the bridged diferrocene compound. After another hour an excess of sodium borohydride in water (25 mM, 30 mL) was dropped into the solution, and the reaction mixture was stirred for an additional hour at room temperature. DMF and water were evaporated under reduced pressure and the residue was extracted several times with ether and water. The ether extract was evaporated to dryness.

Synthesis of the redox polymer was done as follows: 20 mg of each copolymer was soaked and swollen in a Na-HEPES buffer solution (30 mL) and an excess of the ferrocene amine derivative was added. The water-soluble carbodiimide was added to small portions during the first hour. The reaction was allowed to proceed over four days. The polymer was purified using cellulose dialysis tube and freeze dried. The synthesized polymer was crashed in a mortar.

Table 1 shows the elemental ratio of the redox polymers resulting from XPS analysis. Considering from oxygen content, unreacted acrylic acid is presumed to remain.

2.3 Electrode preparation

Glassy carbon disk electrodes (3 mm diameter, Tokai Carbon) were used throughout the experiment. The electrode was polished with waterproof emery paper (#1000, Marumoto), sonicated, rinsed with water and dried. Mixture of the redox polymer and GOD in phosphate buffer solution was dropped onto the electrode surface, and the electrode was exposed to glutaraldehyde vapor to immobilize the polymer and GOD by the crosslinking reaction. Ratio of the redox polymer and GOD was 1:1 in dry weight. Similarly, the electrode modified with the mixture of the redox polymer, GOD and BSA was prepared with glutaraldehyde. Ratio of the

redox polymer, GOD and BSA was 1:3:5 in dry weight.

2.4 Electrochemical measurement

A three-electrode configuration was used throughout. A platinum auxiliary electrode and a silver silver chloride reference electrode were employed. All potentials herein are quoted with respect to it. Electrochemical measurement was carried out in a deoxygenated phosphate buffer solution (0.1 M, pH 7.0) with a computer-controlled potentiostat (EG&G263A, BAS50W).

Hydrodynamic amperometry at fixed potential, 600 mV, was carried out with a rotating disk electrode system (BAS). The enzyme electrode was rotated at 1,000 rpm. After the background current was stable, 50 μ L of 1.0 M glucose solution was successively added to 50 mL of phosphate buffer solution (0.1 M, pH 7.0). The steady-state current response was measured.

3 RESULTS AND DISCUSSION

3.1 Effect of BSA addition on redox stability

The enzyme electrode was fabricated with the mixture of RP-1 (acrylamide : acrylic acid = 4:1) and GOD to be crosslinked by glutaraldehyde treatment, and used for cyclic voltammetry. The typical electrochemical response of the ferrocene-based redox polymer was obtained initially after contact with an electrolyte solution. Continuous soaking in the electrolyte, however, reduced the redox current, and no redox responses were observed in 3 - 6 hours. Typical time course of the anodic peak current (i_{pa}) was shown in Fig. 2. It also demonstrates that drying the electrode at room temperature reactivated it to show higher response than the initial condition. These results

indicate swelling of the polymer hydrogel occurred in aqueous solution, and therefore, distance between the redox sites was stretched. That is why the redox current response of the electrode without BSA was reduced.

Fig. 2 Time course of anodic peak current values of enzyme electrodes co-immobilized with RP-1 in the absence (O) and presence (●) of BSA. The enzyme electrode without BSA was dried at room temperature between 180 and 240 min. Cyclic voltammetry was carried out at scan rate of 30 mV/s. The electrodes were prepared by pipetting of a 5- μL aliquot of polymer-protein mixture. Ratios of the mixtures were RP-1: GOD: BSA = (O); 1: 1: 0, (●); 1: 3: 5 in dry weight.

Addition of BSA for immobilization of the polymer and GOD on redox stability was examined. It was found that immobilization using BSA made the redox response stable after soaking in the electrolyte solution for 2 - 4 hours, as shown in Fig. 2. It was suggested that crosslinking reaction based on BSA prevented the polymer matrix from over-swelling.

3.2 Enzyme electrode co-immobilized with redox polymer and BSA

Figure 3 shows cyclic voltammograms of

the enzyme electrode using BSA at four hours later after soaking it in solution. Linear correlation between i_{pa} and square root of scan rate ($v^{1/2}$) was obtained. Therefore, diffusion-controlled electron transfer apparently takes place between the redox centers of the polymer. From the slope of the plot, the apparent value of $D_R^{1/2}C_R^*$ was estimated to be 4.3×10^{-9} mol $cm^{-2} s^{-1/2}$, where D_R and C_R^* stand for apparent diffusion coefficient of the charge transfer and concentration of the redox center, respectively. This value is the similar level to the previous works^{5,6}. Standard redox potential of the redox polymer was 394 mV. A potential peak-to-peak separation (ΔE_p) was 68 mV at scan rate of 1 mV/s.

Fig. 3 Cyclic voltammograms of enzyme electrode co-immobilized with RP-1 and BSA. Cyclic voltammetry was carried out at scan rate of (a) 30, (b) 20, (c) 10, (d) 5 and (e) 1 mV/s. The electrode was prepared by pipetting of a 5- μL aliquot of 450 mg/mL solution of polymer-protein mixture. Ratio of the mixture was RP-1: GOD: BSA = 1: 3: 5 in dry weight.

Glucose catalytic electrochemistry of the

enzyme electrode was investigated. Figure 4 shows cyclic voltammograms of the enzyme electrode at various glucose concentrations. It demonstrates that catalytic current was observed by addition of glucose and saturated at 7 mM glucose concentration. A shoulder wave was observed at around 320 mV at 3 mM glucose concentration, and no current increase was significantly obtained up to 2 mM. These are probably ascribed that due to depletion in glucose concentration at the vicinity of the electrode, recycling rate of the redox centers was reduced. This phenomenon may be expected in the case at low substrate, high mediator concentrations and high catalytic activity, and this deduction can be supported by the cyclic voltammetric digital simulation in the case of the electrochemical reaction coupled with mediated enzyme reaction¹²). However, we cannot completely deny influence of residual dissolved oxygen as the reason for no current increase up to 2 mM.

Fig. 4 Catalytic electrochemistry of GOD-immobilized electrode with RP-1 and BSA at various glucose concentrations; (a) 0, (b) 3 and (c) 7 mM. The same electrode as Fig. 3 was employed, and potential was scanned at 1 mV/s.

3.3 Effect of ferrocene content

Electrochemical behavior of the enzyme electrode co-immobilized with the redox polymer at various ferrocene contents was investigated by cyclic voltammetry. Correlation between i_{pa} and $v^{1/2}$ was plotted for various redox polymers. From the slope, the apparent values of $D_R^{1/2}C_R^*$ for RP-2, RP-3 and RP-4 were estimated to be 2.4×10^{-10} , 1.2×10^{-9} and 6.2×10^{-10} mol cm⁻² s^{-1/2}, respectively. These values are smaller than that of RP-1. This is likely because small amount, *ca.* 1 μ L, of the hydrogel was loaded for these three electrodes, whereas 5 μ L gel mixture of RP-1 was loaded on the electrode. Furthermore, ΔE_p values at scan rate of 2 mV/s were about 60 mV for these three electrodes. Since ΔE_p was excessively small, the electrochemical kinetics is likely controlled not only by diffusion process. Therefore, electron transfer of these three electrodes was suggested to be transitional condition between semi-infinite diffusion and surface redox processes.

Catalytic electrochemistry for glucose oxidation was investigated by cyclic voltammetry. Catalytic currents were obtained for the enzyme electrodes with RP-2 and RP-3, however, no current increase was observed with RP-4. Hydrophobicity of RP-4 was too high to be homogenized with GOD and BSA, and ferrocenes were rarely able to contact with enzyme molecules. It is known that concentrated acrylic acid produces a hydrophobic polymer¹³). A shoulder was also observed at 2 mM glucose concentration for the enzyme electrode with RP-3, which showed the highest redox activity. No shoulder was observed with RP-2, and this is probably attributed that substrate diffusion to the electrode surface was fast owing to the thin film and that fast redox recycling took place. Figure 5 shows correlation between catalytic current and glucose concentration and Fig. 6 shows plots of ratio of catalytic current to current without substrate, i_{cat}/i_{redox} , vs. glucose concentration. Catalytic current with RP-3 was more than twice as great as with RP-2, whereas i_{cat}/i_{redox} with RP-3 was found to be smaller because i_{redox} with RP-3 was five times greater. This indicates that due to the fact that the enzyme activity is

unproportional to mediator concentration, redox cycling of RP-2 was more effectively used than RP-3.

Fig. 5 Correlation between catalytic current and glucose concentration. The electrodes were prepared by pipetting of a 1- μ L aliquot of 450 mg/mL solution of polymer-protein mixture. Ratio of the mixture was polymer: GOD: BSA = 1: 3: 5 in dry weight. RP-2 (■) and RP-3 (●) were examined. Potential was scanned at 2 mV/s.

Fig. 6 Correlation between i_{cat}/i_{redox} and glucose concentration. The i_{cat}/i_{redox} values were calculated from Fig. 5. RP-2 (■) and RP-3 (●) were examined.

3.4 Rotating disk electrode study for steady-state current response

Steady-state current response at fixed potential was investigated using rotating disk electrode. Figure 7 shows correlation between steady-state current response and glucose concentration. The response of the enzyme electrode with RP-3 was obtained to be greater than RP-2, and the enzyme electrode loading larger amount of the enzyme gel revealed higher current response. The sigmoidal shapes are demonstrated in these glucose calibration plots, and likely because at low glucose concentration, glucose may be consumed in the vicinity of the hydrogel-solution interface, and the concentration gradient of reduced form of ferrocene at the electrode surface did not increase drastically. Therefore, The enzyme electrode that loads a thick enzyme membrane with high enzyme and redox activities may strongly show this behavior. We believe that this is not due to the residual dissolved oxygen, because this behavior can be observed in thoroughly nitrogen-saturated solution. We are in progress to carry out the digital simulation in this case.

Fig. 7 Glucose calibration plots using rotating enzyme electrodes. Ratio of the polymer-protein mixture was redox polymer: GOD: BSA = 1: 3: 5. The electrodes were prepared by pipetting an aliquot of 450 mg/mL solution of polymer-protein mixture. A 1- μ L mixture of RP-2 (○), a 1- μ L mixture of RP-3 (●) and a 2- μ L mixture of RP-3 (□) were loaded on electrodes.

4 CONCLUSIONS

In this work, electron transfer between a ferrocene-based redox polymer and active site of GOD is demonstrated. Crosslinked copolymers of acrylamide and acrylic acid with flexible and long side chains containing ferrocene at the ends were synthesized. GOD and this redox hydrogel were co-immobilized on a glassy carbon electrode surface using glutaraldehyde and BSA. Immobilization based on BSA was highly effective to stabilize electrochemical response of the redox polymer. Addition of BSA for enzyme immobilization prevented over-swelling of the polymer-enzyme hydrogel. Four copolymers with different ferrocene contents were prepared, and electron transfer process of each polymer were explored. The $D_R^{1/2}C_R^*$ values of around 10^{-9} mol cm⁻² s^{-1/2} were obtained. Electrochemical behavior of the redox polymer was found to be almost based on semi-infinite diffusion process. However, thin hydrogel demonstrated the intermediate behavior between surface redox and diffusion-controlled processes. Catalytic electrochemistry of the enzyme electrodes was investigated. The enzyme electrode with high ferrocene content revealed high catalytic current. On the contrary, low catalytic efficiency, i_{cat}/i_{redox} , was observed. In this paper, we proposed new fabrication method for enzyme electrodes with a redox polymer that requires appropriate hydrophilicity. It is anticipated that this technique can be modified for practical use of glucose measurement without considerable difficulty.

The authors acknowledge Dr. Toshiharu Teranishi for helping XPS analysis and Prof. Eiichi Tamiya for helpful discussion. The present work was financially supported by a Grant-in-Aid for

Scientific Research on Priority Areas, No. 236 New Development of Organic Electrochemistry, from the Ministry of Education, Science and Culture, Japan.

REFERENCES

- 1) B. A. Gregg and A. Heller, *Anal. Chem.*, **62**, 258 (1990).
- 2) T. J. Ohara, R. Rajagopalan and A. Heller, *Anal. Chem.*, **65**, 3512 (1993).
- 3) T. J. Ohara, R. Rajagopalan and A. Heller, *Anal. Chem.*, **66**, 2451 (1994).
- 4) E. J. Calvo, C. Danilowicz and L. Diaz, *J. Electroanal. Chem.*, **369**, 279 (1994).
- 5) E. J. Calvo, C. Danilowicz and L. Diaz, *J. Chem. Soc. Faraday Trans.*, **89**, 377 (1993).
- 6) H. Bu, S. R. Mikkelsen and A. M. English, *Anal. Chem.*, **67**, 4071 (1995).
- 7) P. D. Hale, T. Inagaki, H. S. Lee, H. I. Karan, Y. Okamoto and T. A. Skotheim, *Anal. Chim. Acta*, **228**, 31 (1990).
- 8) T. Tatsuma, K. Saito and N. Oyama, *Anal. Chem.*, **66**, 1002 (1994).
- 9) H. Nagasaka, T. Saito, H. Hatakeyama and M. Watanabe, *Denki Kagaku*, **63**, 1088 (1995).
- 10) R. Mosbach, A.-C. Koch-Schmidt and K. Mosbach, *Methods in Enzymology*, Ed. K. Mosbach, **44**, 53 (1976).
- 11) W. Schuhmann, T. Ohara, H.-L. Schmidt and A. Heller, *J. Am. Chem. Soc.*, **113**, 1394 (1991).
- 12) K. Yokoyama, *Abstract of the 63th Annual Meeting of the Electrochemical Society of Japan*, p303 (1996).
- 13) T. Araki, *Gousei Koubunshi III*, Ed. S. Murahashi, M. Imoto, and H. Tani, Asakura Shoten, p219 (1971).