Enzyme Ultra-thin Layer Electrode Prepared by the Co-adsorption of Poly-L-lysine and Glucose Oxidase onto a Mercaptopropionic Acid-Modified Gold Surface

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An ultra-thin layer containing immobilized glucose oxidase was produced through the co-adsorption of the enzyme and poly-L-lysine onto a mercaptopropionic acid-modified gold electrode. The enzyme molecules were considered to be entrapped in a cross-linked matrix consisting of the carboxylic acid on the electrode and the cationic polymer.

The preparation of a thin protein layer onto an electrically-conducting surface is a research area that is useful for a range of applications including the fabrication of bioelectrochemical sensors. A general methodology that can be applied to this purpose is the self-assembly of various functional groups onto specific surfaces such as the reaction of thiols with gold. Jordan *et al.*[1] have reported the formation of a monolayer of positively-charged poly-L-lysine onto a negativelly-charged surface, which is prepared through the self-assembly of 11-mercaptoundecanonic acid on a gold substarte (Figure 1a). On the other hand, we[2] have recently developed a method for immobilizing enzyme molecules into a polyion complex matrix consisting of poly-L-lysine and poly(4-styrenesulfonate) (Figure 1b); an aqueous solution of poly(4-styrenesulfonate) was added to an enzyme/poly-L-lysine mixture and dried to form the enzyme multilayer. By combining the technique for immobilizing enzymes[2] with that for the preparation of the poly-L-lysine monolayer[1], it would be possible to form an enzyme monolayer on a gold surface. That is, a mixture of poly-L-lysine and an enzyme was placed on a negativelly-charged, 3-mercaptopropionic acid monolayer on the gold electrode, so as to form a poly-L-lysine/melcaptopropionic acid matrix with the immobilized enzyme, as shown in Figure 1c. This paper describes preliminary results for the immobilization of glucose oxidase (GOD) and the characteristics of the GOD-based electrode.

A gold electrode (diameter, 1.6 mm; Bioanalytical Systems, West Lafayette, IN) was polished with 0.05 μm alumina and washed with water. The layer of 3-mercaptopropionic acid was prepared onto the gold surface by immersing the electrode in 1 mM (1 M = 1 mol dm^{-3}) ethanolic solution for 1 h at room temperature. After washing the electrode with ethanol and then water, it was immersed in 20 mM potassium phosphate buffer solution (pH 7) containing poly-L-lysine (hydrobromide salt with molecular weight, 90000; Sigma, St. Louis, MO) and GOD [EC 1.1.3.4, from *Aspergillus* sp., 110 U mg^{-1} (which corresponds to 2×10^{10} U mol^{-1}); Toyobo, Osaka] for 24 h at 4 °C. The concentrations of poly-L-lysine and GOD were usually set at 1 mM (per lysine residue) and 0.5 mM, respectively. Then the electrode was thoroughly washed with 0.1 M potassium phosphate buffer (0. 1 M, pH 7). The thiol/GOD/poly-L-lysine-electrode (E 1) thus prepared was stored in 0.1 M potassium phosphate buffer (pH 7) at 4 °C when not in use.

Other GOD-based electrodes were prepared as follows. A GOD-electrode (E 2) and a thiol/GOD-electrode (E 3) were

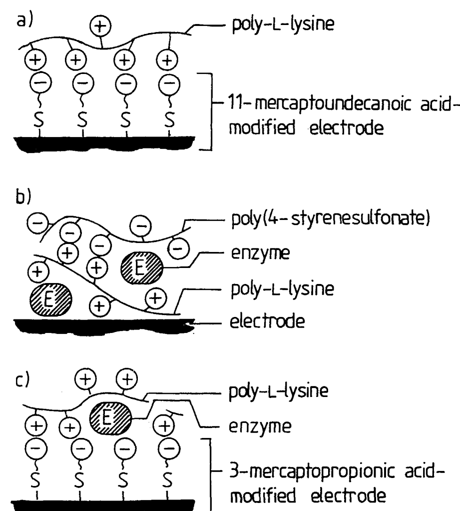

Figure 1. Schematic illustrations of a) poly-L-lysine adsorbed onto a monolayer of 11-mercaptoundecanoic acid formed on a gold electrode, b) enzyme incorporated into a polyion complex layer consisiting of poly-L-lysine and poly(4-styrenesulfonate), and c) enzyme incorporated into a matrix consisting of poly-L-lysine and 3-mercaptopropionic acid on a gold electrode.

made by immersing a bare gold electrode and the thiol-modified one in a 0.5 mM GOD solution without poly-L-lysine (pH 7) for 24 h, respectively, and a GOD/poly-L-lysine-electrode (E 4), by immersing a bare gold electrode in the poly-L-lysine/GOD solution for 24 h. Further, a thiol/poly-L-lysine/GOD-multilayer electrode (E 5) was fabricated by the successive adsorption of poly-L-lysine and GOD on the thiol-modified electrode; the thiol-modified electrode was first immersed in a poly-L-lysine solution (1 mM per lysine residue, pH 7) for 24 h, washed with water and then immersed in a 0.5 mM GOD solution (pH 7). Anionic GOD molecules are expected to be adsorbed onto the polycation layer[3] on the modified electrode.

A potentiostat (HA-150, Hokuto Denko, Tokyo) was used in a three-electrode configuration for amperometric measurements: the modified electrode, an Ag/AgCl reference electrode (saturated with potassium chloride) and a platinum auxiliary electrode were immersed into a test solution (0.1 M potassium phosphate buffer; pH 7, 20 ml, 25.0±0.2 °C) in a cylindrical cell. The potential of the enzyme electrode was set at 0.7 V *vs.* Ag/AgCl. The potential was positive enough to oxidize the hydrogen peroxide produced through the enzymatic reaction. The solution was saturated with air and stirred with a magnetic bar.

The GOD activities were measured by using the peroxidase-phenol-4-aminoantipyrine chromogenic system.[4]

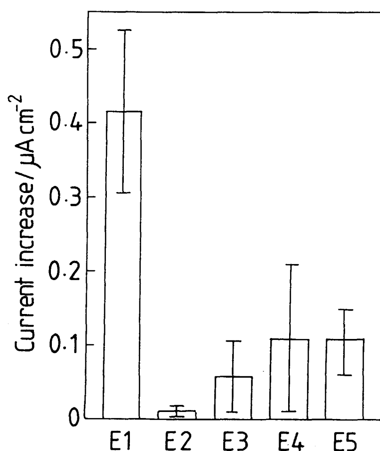

Figure 2. Current responses to 1 mM glucose for E 1-5. Each response was averaged over the measurements by using six different electrodes (n = 6), and standard deviation for the six measurements was shown by bar in the figure.

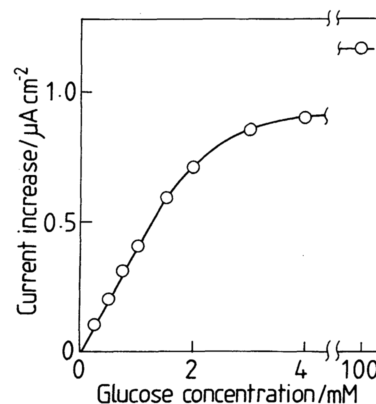

Figure 3. Calibration graph for glucose with E 1.

Figure 2 shows the steady-state current responses to 1 mM glucose for E 1-5. On each electrode, the current increased immediately after the addition of glucose and reached another steady state within 1 s. As each enzyme layer is very thin, the glucose added is expected to diffuse quickly through the layer so as to produce hydrogen peroxide through the GOD reaction, which would result in a fast response on the enzyme electrode. As shown in Figure 2, E 1 gave much larger current response to glucose than other electrodes. The magnitude of glucose response for E1, 0.4 μA cm^{-2} mM^{-1}, was almost the same as that obtained for a GOD monolayer-attached electrode by using avidin and biotinated enzyme.[5]

For E 2, 3 and 5, the current responses to glucose are considered to be caused by the GOD molecules adsorbed on the bare-, thiol modified-, and poly-L-lysine-adsorbed-electrodes, respectively. The weak, nonspecific adsorption on these bare and modified gold surfaces would limit to the amount of the immobilized enzyme, although the poly-L-lysine-adsorbed system (E 5) gave relatively large response among the three electrodes owing to the electro-atractive force between the cationic polymer on the electrode and the negativelly-charged GOD.[3,6] A larger electrode response for E 4 than E 2 is attributable to an increase in the amount of the enzyme adsorbed on the gold surface owing to the formation of some complex between poly-L-lysine and GOD. However, the glucose response for E 4 was still one-fourth of that for E 1. These results indicates that the cross-linked matrix consisting of carboxylate anion on the electrode and poly-L-lysine is effective for immobilizing GOD.

The numbers of enzyme molecules immobilized on the surfaces of E 1, 3 and 5 were estimated by the GOD-activity measurements as follows. The potential of each electrode was set at -1.0 V *vs.* Ag/AgCl for 1 h for the reductive desorption of the thiol compound.[7] This accompanied the dissolution of GOD into the solution; after the reduction, the solution showed GOD activity, whereas the current response to glucose for each electrode reduced to be less than one-tenth of the initial value. GOD activities in the test solution after the cathodic treatment of E 1, 3 and 5 were 1.5, 0.3 and 0.5 mU, respectively. By assuming that the enzyme activity remained unchanged through the immobilization/desorption-process, the amounts of GOD which had been immobilized on the electrode surfaces were calculated to be 3×10^{-12}, 7×10^{-13} and 1×10^{-12} mol cm^{-2} for E 1, 3 and 5, respectively. These results indicate that the magnitude of glucose response (Figure 2) is almost proportional to the surface concentration of GOD for these three electrodes. Further, it seems reasonable that GOD molecules form a monolayer on each electrode; GOD molecule is represented as a sphere of 8 nm diameter,[6] hence the maximum coverage of GOD monolayer is estimated to be 3×10^{-12} mol cm^{-2} (ignoring roughness factor). Results from quartz crystal microbalance measurements also suggested the formation of ultrathin layers, i.e., mono- or bilayers, by the adsorption of GOD or GOD/poly-L-lysine on mercaptopropionic acid-attached surfaces (data not shown).

Figure 3 shows a calibration graph for glucose with E 1. The steady-state current increase for E 1 was proportional to the glucose concentration up to 1.5 mM (27 mg dl^{-1}) . The detection limit was 0.02 mM (0.4 mg dl^{-1}). E 1 could be used for the measurement of glucose for more than a week.

Thus an ultra-thin layer containing GOD was prepared by the co-adsorption of the enzyme and poly-L-lysine onto a negatively-chaeged, thiol-modified electrode. Surface plasmon resonance study on the thiol/enzyme/poly-L-lysine membrane is now in progress. The present method can be applied to the immobilization of a variety of proteins. We have already prepare an L-lactate-sensing electrode by immobilizing lactate oxidase.

References

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