Determination of Enzyme Immobilized into Electropolymerized Polymer Films

Saipin Thanachasai, Hiroyasu Furukawa, Shoichiro Yoshida, and Tadashi Watanabe*
Institute of Industrial Science, The University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153-8505

(Received November 7, 2002; CL-020946)

A simple, rapid and effective way was devised to determine an enzyme incorporated into electropolymerized conductive polymer films, by combining a thin-layer electropolymerization cell and the Bradford Coomassie colorimetric protein assay. The method has been successfully applied to the determination of horseradish peroxidase (HRP) entrapped into pyrrole and 3-alkylsulfonate pyrrole copolymer films.

In designing biosensors, the amount of enzyme confined in the close vicinity of the electrode surface is one of the key parameters which influence considerably the sensor response. However, only few studies have been reported to date on this aspect.[1–8] Previous methods involve radioactive labeling of enzyme,[1–3] the use of a quartz crystal microbalance,[4,5] and the use of surface plasmon resonance to measure the enzyme layer thickness.[6] Additionally, the amount of immobilized glucose oxidase has been determined by stripping the two flavin adenine dinucleotide (FAD) cofactors and subsequently quantifying the detached FAD via either fluorometry or voltammetry.[7,8]

Of these, only the radioactive labeling and the FAD stripping are applicable to an enzyme-encapsulated polymer film on the electrode surface. However, the former is not available in many laboratories, and the latter is restricted to enzymes containing FAD as cofactor. It is thus desirable to develop a general procedure to determine an enzyme entrapped within an electropolymerized polymer film.

If a significant difference of enzyme concentration develops in a monomer solution before and after electropolymerization, the amount of enzyme immobilized into the polymer film can be estimated by a simple protein assay technique. This is attained by use of an easy-to-assemble thin-layer electropolymerization cell, with a sufficiently small quantity of electropolymerization solution against the electrode surface area for electrodeposition. In the present work enzyme electrodes were fabricated using the thin-layer electropolymerization cell depicted in Figure 1, and the well-known Bradford Coomassie dye-binding colorimetric method, with good sensitivity, simplicity and rapidity was adopted for protein assay.[9]

The thin-layer electropolymerization cell was constructed with a tin oxide working electrode, a platinum-black counter electrode and a silicone rubber spacer. An SnO_2-coated glass plate (2.0 cm × 2.5 cm) was pretreated with hot sulfuric acid diluted with water (1:1) for about 30 min and its effective area was 1.0 cm × 2.0 cm after electropolymerization cell assembly. The counter electrode was a platinum plate (2.0 cm × 2.5 cm) with the platinum-black area of 1.0 cm × 2.0 cm. A 2-mm-thick silicone rubber spacer cut out to a similar size as electrode surface was placed between the SnO_2-coated glass electrode and the platinum black electrode to provide an inner volume of 400 μL electropolymerization solution. An aqueous solution (400 μL) containing 0.04 M pyrrole (Py), 0.01 M sodium 4-(3-pyrrolyl)butanesulfonate (PS), and 0.6 g L^{-1} HRP, unless otherwise noted, filled this thin-layer cell. PS was synthesized following a procedure described in the literature,[10] and characterized by NMR (^{1}H NMR (D_2O): δ1.52–1.63 (m, 4H), 2.36 (t, 2H), 2.78 (t, 2H), 5.97 (m, 1H), 6.56 (br s, 1H), 6.66 (m, 1H)). The Py-PS copolymer was chosen here as the enzyme immobilization matrix because of its performance in binding positively charged HRP molecules more strongly than PPy does.[11] Electrochemical polymerization was carried out in a galvanostatic mode at a current density of 0.1 mA cm^{-2}.

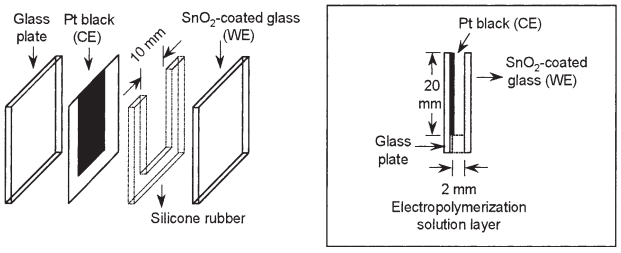

Figure 1. The thin-layer electropolymerization cell.

The enzyme concentration was assayed by monitoring the increased absorbance at 595 nm due to the binding of protein to Coomassie Brilliant Blue (CBB) G-250. The HRP concentration in an aqueous stock solution (2 mg mL^{-1}) was determined spectrophotometrically at 403 nm using an extinction coefficient (ε) of 102 mM^{-1} cm^{-1}.[12] A set of HRP standards (1–30 μg mL^{-1}) was then prepared by properly diluting the stock solution with 1/15 M phosphate buffer (pH 6.4). To each 2 mL of enzyme solution, 2 mL of the CBB G-250 solution was added and after 2 min from mixing the absorbance at 595 nm was measured versus a water reference. The absorbance at 595 nm for each standard was subtracted by the 595 nm reading for a blank prepared from 2 mL of 1/15 M phosphate buffer and 2 mL of the CBB solution. A calibration curve for HRP, being linear up to 30 μg mL^{-1}, was thus obtained.

Preliminary studies were done in regard to the thin-layer cell and the HRP assay. For the thin-layer cell, the area for enzyme immobilization was initially fixed to 2 cm^2 and the thickness of the electropolymerization solution layer was varied. The thickness of 2 mm was optimal for determining the enzyme incorporated in electropolymerized polymer films, due to a compromise between the magnitude of absorbance difference and the initial enzyme concentration drop in the electropolymerization solution. For the HRP assay, the stability of the HRP-CBB complex color, the selectivity and reproducibility were examined. The HRP-CBB complex color was stable for at least 30 min at room temperature, and neither pyrrole nor pyrrole sulfonate (PS) present in the electropolymerization solution interfered with the HRP assay. The relative standard deviation (RSD) ($n = 9$) for a HRP-containing monomer solution was 0.5%. The monomer solution was diluted 20-fold with 1/15 M phosphate buffer (pH

6.4) before measurement to fit to a calibration curve.

The absorption spectra of protein-dye complexes resulting from HRP-containing monomer solutions before and after electropolymerization are given in Figure 2. The inset shows the difference in absorbance at 595 nm from which the quantity of HRP entrapped into the electrosynthesized polymer film was calculated using the calibration curve. In all of the experiments the HRP-containing monomer solutions before and after electropolymerization were assayed in triplicate and an average value of absorbance at 595 nm was adopted to calculate the amount of enzyme.

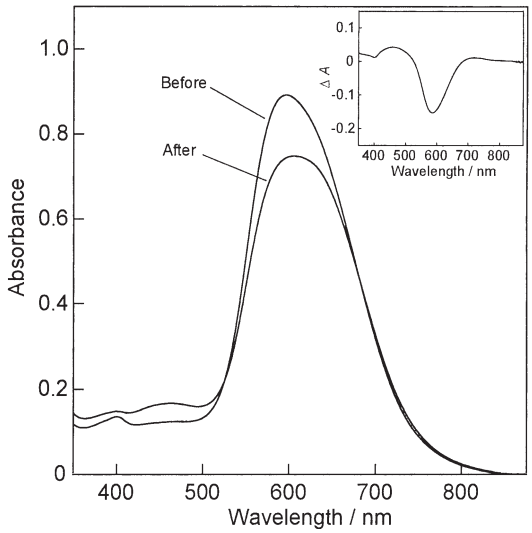

Figure 2. Absorption spectra of HRP-CBB complexes. The samples were HRP-containing monomer solutions before and after electropolymerization.

Figure 3 exhibits the amount of HRP entrapped within the Py-PS copolymer films against the total charge passed, for two HRP concentrations in the electrodeposition solution. As expected, a larger amount of enzyme could be incorporated into the electropolymerized polymer film at a higher enzyme concentration used for film formation. At a fixed HRP concentration (0.1 or 0.6 g L^{-1}) in the growth solution, the amount of HRP entrapped increases almost linearly with the total charge passed up to at least 300 mC cm^{-2}. A similar result has been reported for polypyrrole films with glucose oxidase and hydroquinone sulfonate entrapped.[7] The finding suggests that both the polymer film and enzyme distribution are fairly uniform when prepared by electropolymerization. The rates of enzyme immobilization into the film were 5.91×10^{-14} and 1.74×10^{-13} mol cm^{-2} s^{-1} when electrodeposited at the current density of 0.1 mA cm^{-2} from growth solutions containing HRP 0.1 and 0.6 g L^{-1}, respectively.

The level of enzyme loading, as estimated from Figure 3, was $(5.13 \pm 0.45) \times 10^{-10}$ mol cm^{-2} (95% confidence level, 3 electrodes) for the HRP/Py-PS electrodes prepared with the electropolymerization charge of 300 mC cm^{-2} in the growth solution containing 0.6 g L^{-1} HRP. On the basis of the estimated molar mass of a repeating Py and PS unit in the copolymer, according to elemental analysis results in our earlier study[11] and the assumed density of the copolymer films of 1.2–1.5 g cm^{-3}, referring to the density of polypyrrole and various poly-*N*-alkylpyrrole polymers reported in the literature,[13,14] the electropolymerization charge of

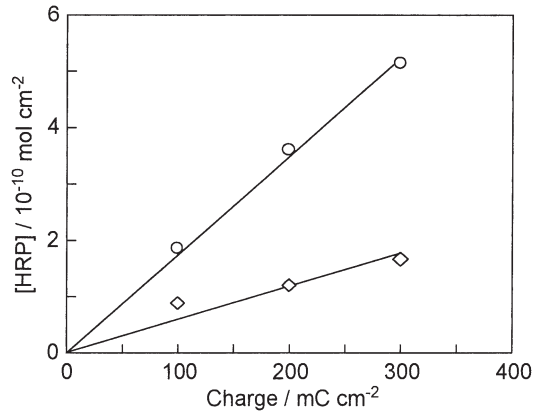

Figure 3. The amount of HRP immobilized in the HRP/Py-PS electrodes as a function of the total charge passed during electropolymerization at HRP concentrations 0.1 g L^{-1} (◇) and 0.6 g L^{-1} (◯) in the growth solution.

300 mC cm^{-2} was estimated[15] to produce a Py-PS film of 1.16–1.46 μm in thickness. The film thickness may have been slightly overestimated since the total charge passed during electrolysis includes a small contribution from the charge associating with electrochemical doping of the polymer. The maximum coverage of the electrode surface with HRP, corresponding to a densely packed layer of HRP that could be viewed as a globule with a diameter of 3 nm,[16] was $7.1–9.0 \times 10^{-9}$ mol cm^{-2}. The above-mentioned value, 5.13×10^{-10} mol cm^{-2}, corresponds hence to an enzymatic surface coverage of 6–7%.

The technique proposed here could be used to clarify one of the most important aspects of an electrochemical biosensor, i.e., the relationship between the sensor performance and the amount of surface-immobilized enzyme molecules.

References

1 P. N. Bartlett, Z. Ali, and V. Eastwick-Field, *J. Chem. Soc., Faraday. Trans.*, **88**, 2677 (1992).
2 I. Willner, A. Riklin, B. Shoham, D. Rivenzon, and E. Katz, *Adv. Mater.*, **5**, 912 (1993).
3 I. Willner and A. Riklin, *Anal. Chem.*, **66**, 1535 (1994).
4 I. Willner V. Heleg-Shabtai, R. Blonder, E. Katz, G. Tao, A. F. Bückmann, and A. Heller, *J. Am. Chem. Soc.*, **118**, 10321 (1996).
5 T. Tatsuma, K. Ariyama, and N. Oyama, *J. Electroanal. Chem.*, **446**, 205 (1998).
6 D. D. Schlereth and R. P. H. Kooyman, *J. Electroanal. Chem.*, **431**, 285 (1997).
7 Y. Kajiya, H. Sugai, C. Iwakura, and H. Yoneyama, *Anal. Chem.*, **63**, 49 (1991).
8 J. J. Gooding, M. Situmorang, P. Erokhin, and D. B. Hibbert, *Anal. Commun.*, **36**, 225 (1999).
9 M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
10 E. E. Havinga, W. ten Hoeve, E. W. Meijer, and H. Wynberg, *Chem. Mater.*, **1**, 650 (1989).
11 S. Thanachasai, S. Rokutanzono, S. Yoshida, and T. Watanabe, *Anal. Sci.*, **18**, 773 (2002).
12 H. B. Dunford and J. S. Stillman, *Coord. Chem. Rev.*, **19**, 187 (1976).
13 M. Salmon, A. F. Diaz, A. J. Logan, M. Krounbi, and J. Bargon, *Mol. Cryst. Liq. Cryst.*, **83**, 265 (1982).
14 A. F. Diaz and K. K. Kanazawa, in "Extended Linear Chain Compounds," ed. by J. S. Miller, Plenum Press, New York (1983), p 417.
15 S. Holdcroft and B. L. Funt, *J. Electroanal. Chem.*, **240**, 89 (1988).
16 E. E. Ferapontova, V. G. Grigorenko, A. M. Egorov, T. Börchers, T. Ruzgas, and L. Gorton, *Biosens. Bioelectron.*, **16**, 147 (2001).