Layer-by-Layer Assembly of Low Molecular Weight Dye/Enzyme Composite Thin Films for Biosensor Appilcation

Yangmei Li,[†,††] Zhichun Chen,[†] Xiuming Jiang,[†] and Xianfu Lin*[†]
[†]*Chemistry Department of Zhejiang University, Hangzhou, Zhejiang, 310027, P. R. China*
[††]*Chemical Engineering Department of Hangzhou Professional Technical College, Hangzhou 310018, P. R. China*

(Received February 4, 2004; CL-040132)

The multilayer films of low molecular weight dye methylene blue (MB) and horseradish peroxidase (HRP) were fabricated by layer-by-layer assembly through electrostatic force. MB was pre-absorbed with anionic polyelectrolyte poly(sodium-*p*-styrenesulfonate) (PSS). The MB/HRP multilayer film-modified Au electrode functioned as a biosensor for H_2O_2.

Enzymatic biosensors are very useful tools in analytical works. A number of approaches to construct amperometric biosensors have been adopted since the conception of the first device by Clark in 1960s. The immobilization of enzyme on the sensing electrode surface is one of the most important points in the design of a stable and sensitive biosensor. Several methods were employed for enzyme immobilization with ordered monolayer or multilayer systems such as Langmuir–Blodgett technique, self-assembly monolayer films, antigen–antibody interactions, and avidin-biotin interactions.[1]

Layer-by-layer assembly, since first introduced by Decher in 1990s to produce nano-thin films by alternative adsorption of polycation and polyanion, has been extended to assemble several materials such as colloids, proteins, clays, and DNA.[2] The major advantages of this novel technique are that many different materials can be incorporated into individual multilayer films as demanded to produce composite materials. This technique also has great potential to produce the reagentless biosensors.[3]

HRP is an enzyme which can catalyze the reaction between hydrogen donor and acceptor. H_2O_2, which can be produced in most enzymatic reactions by oxidase such as glucose oxidase and lactate oxidase, is the most popular hydrogen acceptor.[4] The corporation of HRP and electron mediate together to prepare the biosensor is thus of great importance. MB, which is an organic low molecular weight dye with one positive charge per molecule, can act as an electron mediator to promote the electrochemical reaction between the immobilized HRP and the electrode surface.[5] Several methods have been done to coimmobilize the enzyme and MB together on the electrode such as electrochemical polymerization,[6] immobilization in sol–gel ceramic matrix.[7] Conventional LBL assembly films of MB with negatively charged materials have disadvantages of unstable for lack of positive charges of MB. We report here the method of pre-absorbed of MB on PSS [poly(sodium-*p*-styrenesulfonate)] to preparation of multilayer thin films composed of HRP and MB through LBL assembly on the surface of a gold electrode and their catalytic activity.

The formation of a multilayer films of HRP/MB was preformed on PET [poly(ethylene terephthalate)] and gold substrates, respectively. MB was premixed with PSS. The PSS concentration in the premixed PSS–MB solution was of 1 g·dm^{-3} with the charge proportion of the PSS:MB of 4:1. The surface of PET was first negatively charged with 1.5 mol·dm^{-3} NaOH for 20 min at 40 °C.[8] Then the negatively charged PET was dipped into 0.5 g·dm^{-3} HRP (in 1/15 mol·dm^{-3} phosphate buffered saline (PBS) of pH 6.8) for 40 min. The gold electrode was first dipped into 0.02 mol·dm^{-3} cysteamine hydrochloride overnight to form the self-assembly films (SAMs). These two pretreated substances were immersed into 1 g·dm^{-3} PSS–MB (in 1/15 mol·dm^{-3} PBS of pH 6.8) at 25 °C for 20 min. After being rinsed thrice with PBS, the substrates were immersed in 0.5 g·dm^{-3} (in 1/15 mol·dm^{-3} PBS of pH 6.8) HRP for 40 min. HRP is positively charged at pH 6.8. LBL assembly films of PSS–MB/HRP were constructed on both sides of the substrates by following the above steps. Repeated the above steps gave the multilayer films of PSS–MB/HRP.

UV–vis spectroscopy was used to follow the assembly process of HRP and MB on PET substrates. Figure 1 shows the absorption spectra of 2, 4, 6- and 8- bilayers of HRP/PSS–MB assembled on PET slides, respectively. The absorption peak at 610 nm characters the π–π^* transition of MB. From the insert in Figure 1, it shows that the absorbance increase at 610 nm is linear with the number of assembled MB, which clearly imply that a constant amount of MB was immobilized upon each deposition to form layer-by-layer structures. Using the absorbance values in the 610 nm and the corresponding extinction coefficient 3.55×10^4 L·mol^{-1}·cm^{-1} calculated from the solution absorption spectrum of MB, an average MB surface density of 9.8×10^{-12} mol·cm^{-2} is obtained for each PSS–MB layer of $(\text{PSS–MB/HRP})_n$ multilayer films.

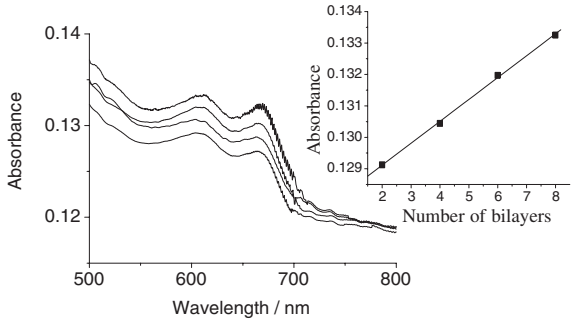

Figure 1. UV–vis absorption spectra of HRP/PSS–MB multilayers with different number of layers on PET slide. From the lower to upper curves, the number of bilayers are 2, 4, 6, 8, respectively. Insert shows the relationship of the absorbance at 610 nm vs the number of HRP/PSS–MB bilayers.

To check whether the ionic strength influence the surface coverage of MB, other two different concentrations of PBS were

used. In 1/40 and 1/80 mol·dm^{-3} PBS, the absorbance of the films with same assembly number of PSS–MB/HRP was smaller than that of the film in the 1/15 mol·dm^{-3} PBS. The lower the ionic strength of the solutions, the less the absorbance is. It indicates that the ionic strength affects the loading of MB in the multilayer films. Large ionic strength of PSS solution helps the polyelectrolyte to form more coiled structure, thus can wrap much more MB molecules in it.

The fabrication of PSS–MB/HRP multilayer films on gold electrode for biosensor was studied. Cyclic voltammetry was used for the electrochemical properties of the films. Figure 2 shows the cyclic voltammograms (CVs) of the four bilayers of PSS–MB/HRP modified electrode at the scan rate of 100 mV·S^{-1} in a solution of 1/15 mol·dm^{-3} PBS containing 0.2 mol·dm^{-3}KCl. CVs show a pair of redox peaks of MB. The anodic/cathodic peak currents increased linearly with the scan rates in the range of 5–200 mV·S^{-1}, indicating the surface electrochemical process of MB.

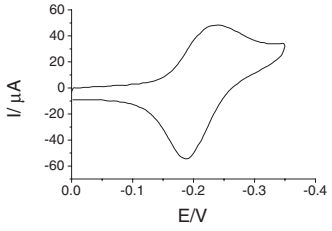

Figure 2. CVs of 4-bilayers of PSS–MB/HRP modified electrode in 1/15 mol·dm^{-3} PBS containing 0.2 mol·dm^{-3}KCl.

The response current of the modified electrodes with different number of PSS–MB/HRP layers was examined in the PBS solution. When the number of the bilayers exceeded three, the CV peak of MB appeared and the modified electrode electrocatalyzed the reduction of H_2O_2. The current of the modified electrodes increased with the number of PSS–MB/HRP layers.

The calibration curve of the catalytic current at -0.25 V vs Ag/AgCl with variations of concentration of H_2O_2 as a function of the number of PSS–MB/HRP layers is shown in Figure 3. Modified with the 3 bilayers of PSS–MB/HRP, the electrode exhibited satisfactory calibration graphs over the concentration range of 0.33–8.82 mmol·dm^{-3} H_2O_2. Modified with the 4 bilayers of PSS–MB/HRP, the linear range of the concentration of H_2O_2 reaches to 14.3 mmol·dm^{-3}. The sensitivity of the biosensors was 1.12, 2.52, and 3.35 mA mol^{-1}·dm^3 for the 3-, 4-, and 5-bilayer PSS–MB/HRP films, respectively. Figure 4 shows the sensitivity of the modified electrodes as a function of the number of PSS–MB/HRP layers. The sensitivity depended linearly on the number of layers, showing that a constant amount of HRP was immobilized upon each deposition. The apparent Michaelis constant (K_m^{app}) for the 3-bilayers was 6.30 and 18.47 mmol·dm^{-3} for the 4-bilayers of PSS–MB/HRP films. These values are larger than that reported by other immobilization method.[9]

The response characteristics of the modified electrode with different bilayers of PSS–MB/HRP as H_2O_2 sensor were evaluated. The modified electrode showed a rapid response to H_2O_2, the response time being 5–7 s for the 3- and 4-bilayers modified electrode. The rapid response of the sensors can be ascribed to the thin nature of the PSS–MB/HRP films.

The stability of the sensor was also evaluated by detecting the response currents in the 1.63 mmol·dm^{-3} H_2O_2 solution. The sensor was repeatedly used for 2 h within one week. It was preserved in refrigerator at 4 °C when not used. The current of blank test dropped to 81% of the original value after one week. The catalytic current was decreased down to about 75% of the original. This response current remained quite stable after fifty days or longer. It means the stability of the sensor is quite good.

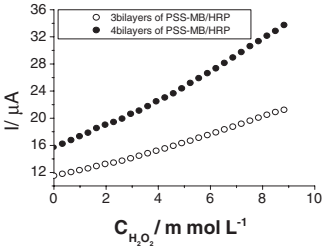

Figure 3. The calibration curves of H_2O_2.

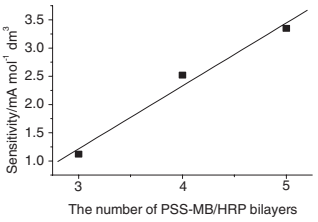

Figure 4. The relationship between the number of bilayers of PSS–MB/HRP and the sensitivity of the modified electrode.

In summary, PSS–MB/HRP multilayer films can be fabricated by simply pre-absorbed low molecular weight dye methylene blue on polyelectrolyte PSS and then alternatively assembled negatively charged PSS–MB and positively charged HRP layer by layer. The resulting multilayer films are useful for the sensoring layer of reagentless H_2O_2 biosensor. The biosensor possesses very good sensitivity, stability and reproducibility. The results also imply that the present procedure may be applied to other enzymes and other small molecular mediators.

This work was supported by International Cooperation Project (021106132) from the Science and Technology Bureau of Zhejiang, China.

References

1 a) J. I. Anzai, Y. Kobayashi, T. Hoshi, and H. Saiki, *Chem. Lett.*, **1999**, 365. b) H. Zimmermann, A. Lindgren, W. Schnhmann, and L. Gorton, *Chem.—Eur. J.*, **6**, 592 (2000). c) T. Hoshi, H. Saiki, and J. I. Anzai, *J. Chem. Soc., Perkin Trans.*, **1999**, 1293.
2 L. X. Shi, Y. X. Lu, J. Sun, J. Zhang, C. Q. Sun, J. Q. Liu, and J. C. Shen, *Biomacromolecules*, **4**, 1161 (2003).
3 A. Narvaez, G. Suarez, I. C. Popescu, I. Katakis, and E. Dominguez, *Biosens. Bioelectron.*, **15**, 43 (2000).
4 Y. Kobayashi and J. I. Anzai, *J. Electroanal. Chem.*, **507**, 250 (2001).
5 Y. M. Li, Z. C. Chen, D. S. Lv, and X. F. Lin, *Wuli Huaxue Xuebao*, **19**, 474 (2003).
6 C. M. A. Brett, G. Inzeltb, and V. Kertesz, *Anal. Chim. Acta*, **385**, 119 (1999).
7 S. B. Khoo and F. Chen, *Anal. Chem.*, **74**, 5734 (2002).
8 W. Chen and T. J. McCarthy, *Macromolecules*, **30**, 78 (1997).
9 J. Z. Xu, J. J. Zhu, Q. Wu, Z. Hu, and H. Y. Chen, *Electroanalysis*, **15**, 219 (2003).

Published on the web (Advance View) April 12, 2004; DOI 10.1246/cl.2004.564