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A Novel Reagentless Biosensor Constructed by Layer-by-Layer Assembly of HRP and Nile Blue Premixed with Polyanion

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Abstract: A novel reagentless biosensor constructed by the organic dye nile blue (NB) and horseradish peroxidase (HRP) has been fabricated *via* layer-by-layer (LBL) self-assembly technique. NB premixed with polyanion poly (sodium-*p*-styrenesulfonate) (PSS) acts as the mediator between the immobilized HRP and the electrode surface. The response of the biosensor to hydrogen peroxide has been investigated. The linear range of the biosensor to hydrogen peroxide was from 0.20 mmol/L to 7.03 mmol /L with a sensitivity of 8.45 μA/(mmol/L).

Keywords: Nile blue, premixed, layer-by-layer, horseradish peroxidase (HRP), biosensor.

Electrostatic layer-by-layer (LBL) assembly was first proposed by Decher in 1990s[1,2]. This film assembly approach has great advantages of the simplicity preparation of ultrathin films with defined composition and uniform thickness in nanoscale. Several attempts have been made to fabricate composite enzyme electrodes[3,4] with the method. Hodak *et al*.[5] introduced LBL assembly technique to construct reagentless biosensor with glucose oxidase (GOD) and ferrocene modified poly(allylamine) polymer. Sun *et al*.[6]and Chen *et al*.[7] fabricated HRP and GOD biosensors with Os-based redox polymer and enzymes. We incorporated organic dye methylene blue with enzyme to fabricate reagentless biosensor[8].

Nile blue, a phenoxiazine dye, which has one positive charge within one molecule, has shown very promising properties as a redox mediator. It was adsorbed on different electrode surfaces for the oxidation of NADH[9,10], or covalently immobilized on gold electrode for the reduction of hemoglobin[11] and HRP[12]. Due to the less charged groups in NB, to assemble it with LBL directly is difficult. Premixing of NB with polyanion *prior to* assembly is introduced to overcome the problem. To our knowledge, it has not been reported that LBL assembled NB premixed with polyanion to fabricate reagentless electrode. In this paper a novel amperometric HRP-modified electrode for the determination of hydrogen peroxidase was constructed by alternatively immobilization of HRP and NB premixed with PSS multilayer films on gold electrode. The response of the biosensor to hydrogen peroxide has been investigated.

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Experimental

The formation of a multilayer films of HRP/PSS-NB was performed on PET [poly(ethylene terephthalate)] and gold substrates respectively. UV-Vis spectroscopy of the multilayer on PET film was investigated. The method of negative ionization of the surface of PET film is the same as that in reference[13]. The negative PET was first dipped into positively charged HRP (0.5 mg/mL) at pH 6.5 in 1/15 mol/L phosphate buffer for 40 min. The gold electrode was cleaned as the reference[14]. Then the cleaned gold electrode was immersed in aqueous solution of 0.02 mol/L cysteamine hydrochloride for 16 h to form a monolayer of positive charge. The two pretreated substrates were dipped into the premixed solution of PSS-NB at 25 ºC for 20 min, which was composed of 1 mg/mL PSS and 0.34 mg/mL NB in pH 6.5 phosphate buffer (1/15 mol/L) containing 0.3 mol/L NaCl. After being rinsed thrice with water, the two substrates were immersed in HRP solution for 40 min. The steps were repeated to obtain HRP/PSS-NB multilayer films.

Results and Discussion

UV-Vis spectroscopy was used to characterize the multilayer films assembled on PET slides. **Figure 1** shows the UV-Visible absorption of 2, 4, 6, and 8 bilayers of PSS-NB/HRP assembled on PET slides. The major characteristic absorption peak at 597 nm was assigned to the π–π* transition of NB. The linear increase of the absorbance at 597 nm indicates that a constant amount of PSS-NB/HRP could be immobilized to form multilayer films.

Cyclic votammetry was used to characterize the electrochemical properties of the PSS-NB/HRP multilayer modified gold electrode. The peak current increased with the number of assembled layers within two bilayers, and leveled off beyond two bilayers. The reason was that thicker layers of assembled film hindered diffusion of the substrate

Figure 1 UV-Vis absorption spectra of PSS-NB/HRP multilayers with different number of layers on PET slides (Inset shows the relationship of the absorbance at 597 nm *vs*. the number of PSS-NB/HRP bilayers)

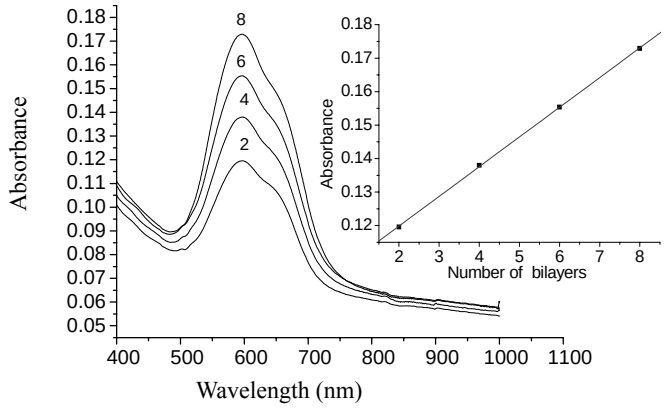
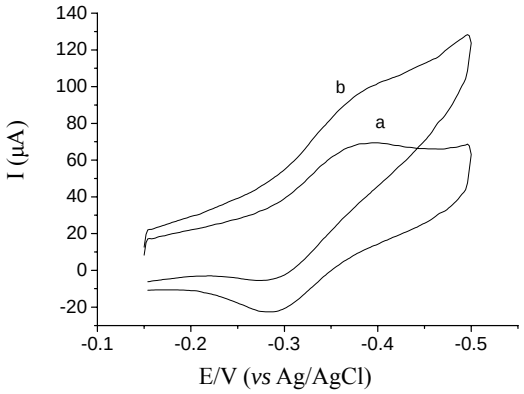


Figure 2 Cyclic voltammograms of the multilayer of PSS-NB/HRP modified electrode (a) in the absence of H_2O_2 and (b) in the presence of 1.31 mmol/L H_2O_2. Condition: in 1/15 mol/L phosphate buffer (pH=6.8), scan rate 100 mv/s.

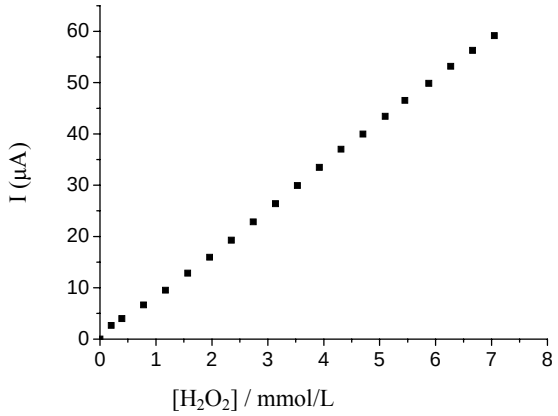


beyond two bilayers. Thus, we investigated the modified electrode containing two bilayers of PSS-NB/HRP. The plot of cathodic/anodic peak currents *vs.* the square root of scan rate showed a good linear relation in the range of 20-150 mV/s, which was a diffusion-controlled electrode process. The catalytic reduction of H_2O_2 at the two bilayers films containing HRP was clearly observed in **Figure 2**. After adding H_2O_2, a typical catalytic reduction wave could be observed, which was an increase in cathodic currents and a concomitant decrease in anodic currents in cyclic voltammograms. It appeared that NB could act as an efficient electron transfer relay system between the active sites of HRP and the electrode surface.

The electrode response to H_2O_2 increased with the change of applied potential from 0 to -0.4 V *vs* Ag/AgCl. Taking into account the sensitivity and interference, the potential of -0. 3 V was selected. The calibration plot of catalytic current with various concentrations of H_2O_2 is given at -0.3 V *vs* Ag/AgCl in **Figure 3**. The linear range of H_2O_2 was from 0.20 mmol/L to 7.03 mmol /L with a sensitivity of 8.45 μA/(mmol/L) and a correlation coefficent of 0.999. The detection limit of modified electrode was 0.03 mmol /L at a signal-to-noise ratio of 3. The apparent Michaelis-Menten constant K_M^{app} was calculated to be 4.90 mmol/L. The stability of the multilayer electrode was also evaluated by monitoring the response currents in the presence of 1.96 mmol/L H_2O_2. The electrode was used once a week. It was stored at 4 ºC in a refrigerator when not used. After three weeks the response was preserved to be about 74%. Compared with other method of biosensor construction, the stability of the sensor is quite good[12].

In conclusion, a novel reagentless amperometric HRP biosensor was constructed by LBL assembly of HRP and NB premixed with PSS. NB described in this work effectively mediated the electron transfer from HRP to the gold surface. The assembled enzyme electrode can be applied to catalyze the reduction of H_2O_2 and exhibits simple fabrication, good sensitivity and stability.

Figure 3 Calibration plot of the multilayer electrode, obtained at -0.3 V in 1/15 mol/L phosphate buffer (pH 6.8)



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