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Electrochemical surface plasmon resonance detection of enzymatic reaction in bilayer lipid membranes

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Abstract

In this paper, electrochemical surface plasmon resonance (SPR) method was first used to detect enzymatic reaction in bilayer lipid membrane (BLM) based on immobilizing horseradish peroxidase (HRP) in the BLMs supported by the redox polyaniline (PAn) film. By SPR kinetic curve in situ monitoring the redox transformation of PAn film resulted from the reaction between HRP and PAn, the enzymatic reaction of HRP with H_2O_2 was successfully analyzed by electrochemical SPR spectroscopy. The results show that this BLM supported on PAn film cannot only preserve the bioactivity of HRP immobilized in the membrane, but also provide a channel for the transfer of electrons between HRP and PAn on electrode surface. These characteristics enabled the development of SPR biosensor for sensitively detecting H_2O_2. H_2O_2 has been detected by electrochemical SPR spectroscopy in the concentration range of 5×10^{-5} M to 2×10^{-3} M. After each of detections, the SPR sensor surface was completely regenerated by electrochemically reducing the oxidized PAn to its reduced state. This method provides a novel route for enhancing the detection of small ligand of enzymatic reaction in BLM by electrochemical SPR spectroscopy.

Keywords: Electrochemical surface plasmon resonance; LBM; Enzymatic reaction

1. Introduction

Optical surface plasmon resonance spectroscopy is a powerful tool for in situ real-time characterization of solid/liquid interfaces [1]. This technology has been widely used for the study of interactions of biological molecules because of its characters as a rapid, label-free, high selective and high sensitive assay method [2–7]. However, these researches mainly focus on studying the binding process between the large biomolecules, the reaction process between the large biomolecules and their small ligands are seldom detected by SPR spectroscopy, because the changes in the refractive index resulted from the binding of small ligand are very small. Some methods have been used to improve the sensitivity of SPR for detecting the reaction of these small ligands or other small molecules. The first method employed polymers with high molecular weights as a transduction layer to increase the changes in the refractive index at the sensing interface triggered by small molecules [8]. The second method utilized the optoelectronic response of inorganic material to enhance SPR signal for detection of small biomolecules [9,10]. The third method reported in literature utilized the conformational change of macromolecules or proteins switched by small molecules to amplify the change of SPR signal [11,12]. The fourth method was the use of a high molecular weight ligand that competing with the small molecular weight analyte for receptor binding [13]. Here, we propose a novel way by utilizing electrochemical SPR spectroscopy [14] to detect an enzymatic reaction in BLM.

As well known, HRP is able to oxidize PAn film without requiring the intervention of any additional mediator species [15]. This enabled the development of electrochemical biosensor for detecting H_2O_2. A serious problem in the application of this system is how to immobilize enzyme on the surface of electrode with high stability and enzymatic activity. It has been confirmed that modification of electrode by lipid can substantially preserve the bioactivity of enzyme and improve the sensitivity and stability of a biosensor because of the characteristic of BLMs [16]. The heme proteins have been embedded in

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BLMs and the corresponded biosensors have been constructed [17]. However, these biosensors were mainly characterized by electrochemistry, electrochemical SPR spectroscopy has never been used to detect the capability of these biosensors constructed by embedding enzyme in BLMs for the binding of small ligands with the enzyme. Herein, we prepared a BLM containing HRP on a SPR gold substrate modified by PAn film, and investigated the preparation process of this composite film by SPR spectroscopy. Furthermore, we evaluated the enzymatic reaction between HRP and H_2O_2 by electrochemical SPR spectroscopy.

2. Experimental

2.1. Reagents

Aniline (Beijing Chemical, 99.7%) was used after distillation, citric acid (99.5%) and H_2O_2 (30%) (Beijing Chemical) were used without further purification. Horseradish peroxidase (HRP) was purchased from Beijing Dingguo Biotechnology Development Center. 1,2-Distearoyl-sn-glycerol-3-phosphoglycerol, sodium salt (DSPG) was obtained from sigma and was used as received. Stock solutions of H_2O_2 with different concentrations were freshly prepared each day. All solutions were made with deionized water, which was purified with a Milli-Q system (Millipore).

2.2. Preparation of the enzyme membranes

PAn film was deposited by cycling the potential between −0.2 and 0.9 V with a scan rate of 0.02 V/s in 0.05 M aniline of 0.1 M H_2SO_4 solution. After a cyclic potential was performed, another cyclic scan was followed and the anodic limit was adjusted to 0.8 V to lower the production of unwanted byproducts. The thickness of PAn film measured was 10.5 nm. Vesicles doped with HRP were prepared by dissolving HRP into the 0.50 mg/ml DSPG vesicle solution with a final concentration of 2 mg/ml. The dispersion obtained was coated over the PAn film in a Teflon cuvette at room temperature for 2 h. Finally, the resulted enzyme membrane was washed with acetate buffer solution of pH 5.4, and stored in the buffer solution.

2.3. In situ SPR electrochemical measurements

SPR measurements were performed with a home-built electrochemical SPR system [18]. The SPR-active substrates were prepared by sputtering of gold (∼42.7 nm) to the surface of glass slide that was modified with an underlayer of chromium (∼1.5 nm). For further experiments, SPR-active gold film cleaned through piranha solution was pressed onto the base of a half-cylindrical lens via index matching oil. The gold surface of the glass slide mounted against the Teflon cell with use of a Kalrez O-ring was used as SPR sensor surface and also a working electrode (the apparent electrode area was 0.38 cm^2). The Teflon cell allowed for simultaneous recording of SPR and electrochemical data and applying of a voltage to the sample.

2.4. Electrochemical experiments

Electrochemical experiments were carried out with a CHI800 electrochemical system in a Teflon cell. The Teflon cell was provided with a saturated Ag/AgCl reference electrode and a platinum counter electrode. Electrochemical measurements were all recorded and reported vs. the KCl-saturated Ag/AgCl reference electrode. All experiments were done at room temperature. Buffers were purged with highly purified nitrogen for at least 20 min prior to a series of experiments.

3. Results and discussion

3.1. Electrochemical SPR spectroscopy characterization of the enzyme membrane preparation

In our previous work, we have confirmed that conducting polymer film can be served as a strong support for s-BLM [19]. Here, we further utilized electrochemical SPR spectroscopy to detect enzymatic reaction in BLM based on electron communication between the enzyme immobilized in BLM and PAn. The configuration of enzyme membrane and the reaction mechanism of this enzymatic reaction were shown in Fig. 1. In detail, PAn film was firstly electrodeposited on the surface of SPR gold film. And then, the DSPG bilayer lipid membrane containing HRP was formed on the surface of PAn film by vesicles fusion.

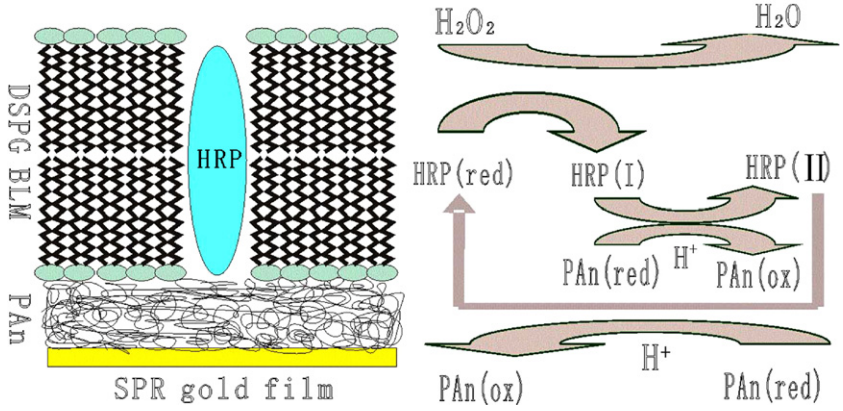

Fig. 1. The configuration and the reaction mechanism of this biosensor for detecting surface enzymatic reaction.

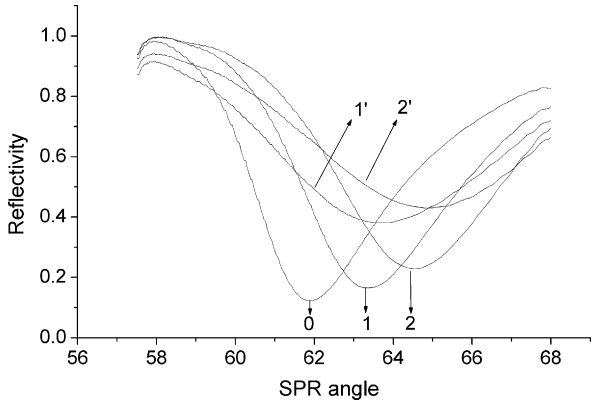

Fig. 2. SPR reflectivity and minimum reflectivity angle curves in situ recorded after various surface modification steps. (0) The bare SPR gold film. (1) The R–θ curve of the PAn film at reduced state. (1′) The R–θ curve of the PAn film at oxidized state. (2) The changes of SPR R–θ curve after BLM containing HRP were deposited on the surface of PAn film (at reduced state). (2′) The changes of SPR R–θ curve after BLM containing HRP were deposited on the surface of PAn film (at oxidated state).

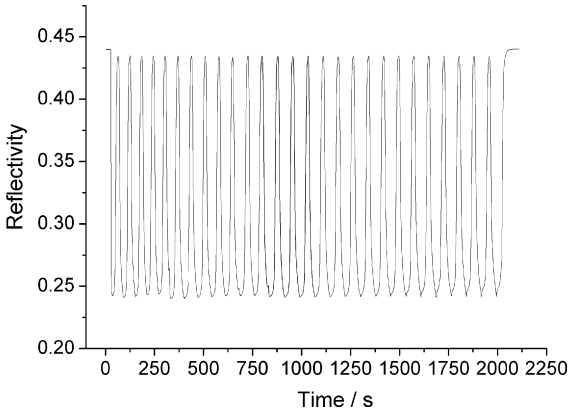

Fig. 3. The change of SPR kinetic curve during continuously electrochemically scanning in pH 5.0 acetate buffer. The angle of incidence was fixed at 65°.

The preparation process of the enzyme membrane was in situ monitored by SPR R–θ curves. As shown in Fig. 2, the R–θ curve of bare Au was recorded as curve 0. The minimum of the R–θ curve shifts to higher angle after PAn film was electrodeposited on SPR gold film. The R–θ curves of the PAn film before and after electrochemical oxidation were recorded as curve 1 and curve 1′, respectively. Compared curve 1 and curve 1′, the SPR R–θ curve for the oxidized PAn film shows a slight shift to higher angle relative to that of the reduced PAn film, but a very distinct change occurs in the resonance depth of SPR curve. This change should be ascribed to the change of PAn film in the optical property resulting from the conductivity change of the PAn film. Many works had discussed the conductivity change of PAn film with potential in a buffer solution of pH 5, and demonstrated that there exists a fast and reversible transition in the conductivity around +0.28 V corresponding to a redox transition between a conducting state, emeraldine, and an insulating state, pernigraniline [20]. Curve 2 shows the change of SPR R–θ curve after BLM containing HRP was deposited on the surface of PAn. The SPR angle increased about 0.88° obtained by the Software fitting. In order to confirm the formation of BLM, we further simulated the thickness of BLM by nonlinear least-squares method. At a wavelength of 670 nm, the refractive index values used for the SPR modeling calculations are 1.61 and 1.3301 for BaK4 lens and water, respectively. The real part of the refractive index used for bulk gold film is 0.1288, and the imaginary part of the refractive index is 3.8627. The thickness of bulk gold film is estimated as 42.7 nm. The optical constants PAn film is known as 1.51 [21] and its thickness is estimated as 10.5 nm. The refractive index of the lipid is about 1.45 [22]. Based on these parameters, the thickness of BLM is estimated as 9.5 nm. This value is larger than the predicted thickness for a planar bilayer (roughly 5 nm thick). The main reason is originated from the mixed morphology of the lipid structure deposited on the surface of PAn film. According to the results reported by Cheng et al. [23], the composite structure consisting of a bilayer, vesicle fragments, and lipid junctions can be formed on some patterned pocket or substrate. The thickness of BLM possessing this structure is larger than a general BLM, and this BLM is not highly insulating. We also observed this character in our experimental results by electrochemical SPR spectroscopy through analyzing the redox change of PAn film before and after BLM deposited. Curve 2 and curve 2′ are the SPR R–θ curve for the reduced and oxidated PAn after BLM containing HRP was deposited. In general, the formation of close packed BLM would strongly block the transfer of electrons and protons between the substrate electrode and solution. So the number of protons needed under the redox change of PAn film would be limited by the ion transfer ability of BLM deposited on the surface of PAn film. Thus, the change of SPR reflectivity minimum between the reduced and oxidized PAn film would disappear if a close packed BLM was formed. However, we can see an obvious change of SPR reflectivity minimum in curve 2 and curve 2′. This change is slightly smaller than that of the value at simple PAn film shown in curve 1 and curve 1′. This minor change in the resonance depth maybe results by an increase in the extinction coefficient k, a measure for the absorptivity of a medium. Since the BLM are non-absorbing at 670 nm, we attribute the apparent increase in k to scattering effects [22]. This is likely caused by the insertion of HRP into the membrane. These results confirmed that this composite film is not highly insulating. This low-insulating character can be also observed from the SPR kinetic curves during the modulation process between the state of the reduced and oxidized PAn film. Fig. 3 is the change of SPR kinetic curve during continuously electrochemically scanning in acetate buffer of pH 5.0. We can see that the reflectivity is changed from 0.24 to 0.44 with the potential scanning from 0 to 0.34 V. It shows that the proton communion between PAn film and solution is produced during the process of potential scanning. We can also find amplitude of potential-modulated reflectivity minimum does not change during the process of scanning. It means this mixed membrane supported on PAn film surface is rather stable, and the SPR sensor surface was completely regenerated by electrochemically reducing the oxidized PAn to its reduced state.

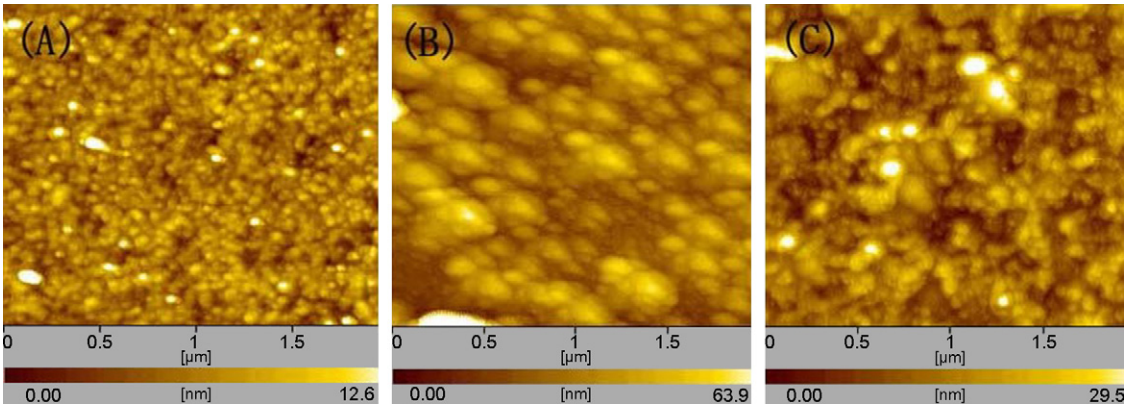

Fig. 4. Typical AFM images of the bare gold (A), the electropolymerized PAn layer (B) and PAn supported BLM containing HRP (C).

3.2. AFM characterization of the enzyme membrane morphology

Except for SPR method, AFM was also used to character the formation process of this enzyme membrane. As convinced by AFM, the gold surface seemed to be smooth with a low RMS roughness (about 1.179 nm) in Fig. 4A. The RMS roughness was increased to 10.08 nm as PAn film with a globular morphology was formed on the surface of gold substrate (Fig. 4B). The increase of rms roughness mainly comes from the uneven growing of PAn film. On the other hand, the self-assembly of DSPG induced a smoother topography than that of the PAn film with rms roughness decreasing from 10.08 to 5.213 nm (Fig. 4C), showing the deposition of BLM onto the PAn surface across the PAn film.

3.3. Electrochemical SPR detection of enzymatic reaction between HRP and H_2O_2

Previous works demonstrated that HRP could reconstitute into lipid bilayer and showed its expected bioactivity for electrocatalyzing H_2O_2. However, this process is difficult to be detected by SPR spectroscopy because the refractive index change in the reaction process between HRP and H_2O_2 is quite small. Therefore, in our case, PAn film is used as underlying substrate for supporting BLM. If HRP is reconstituted into the BLM and contacts with PAn film, the enzymatic reaction of HRP with H_2O_2 should be observed by detecting the redox transformation of PAn film resulting in the electron exchange between HRP and PAn [15]. SPR kinetics curve is used to investigate the catalytic responses of this enzyme substrate to H_2O_2 in solution. In order to confirm the change of SPR response is resulted from the enzymatic reaction between HRP and PAn in the presence of H_2O_2, the comparison experiments containing the reactions of PAn film with H_2O_2 and PAn film supported BLM with H_2O_2 were carried out, respectively. The angle shifts in R–θ curves were not observed in these two experimental conditions (the curves were not shown). Fig. 5 depicts the SPR kinetics curves at different H_2O_2 concentrations. In the presence of H_2O_2, the reflectivity remains unchanged because no redox transformation of PAn film occurred. After adding H_2O_2, the reflectivity would change with the concentrations of H_2O_2. When a lower concentration of H_2O_2 is used, a smaller slope of SPR kinetics curve is obtained, and it means a smaller transformation rate. From Fig. 5, we also observe the response time of the enzymatic reaction for H_2O_2. It should be pointed out that the response time (~350 s) in present system is slower than the results reported by Iwasaki et al. (~100 s). This is because the number of protons needed by the redox change of PAn film is limited by the ion transfer ability of BLM deposited on the surface of PAn film. Although this BLM is none highly insulating, it produce a block effect for proton transfer between PAn and solution. That is why the response time of the SPR biosensor is longer than the other SPR biosensors. From another point of view, this longer response time is in favor of extracting the accurate slope of SPR kinetic curves. A good linear relation between H_2O_2 concentrations and the slope of SPR kinetic curves is obtained. As shown in the inset of Fig. 5, H_2O_2 was successfully detected in the concentration range of 5×10^{-5} M to 2×10^{-3} M. After each of detections, the SPR sensor surface was completely regenerated by electrochemically reducing the oxidized PAn to its reduced state. These results demonstrate that SPR technology combined with electrochemical method is a good technique to investigate enzymatic reaction.

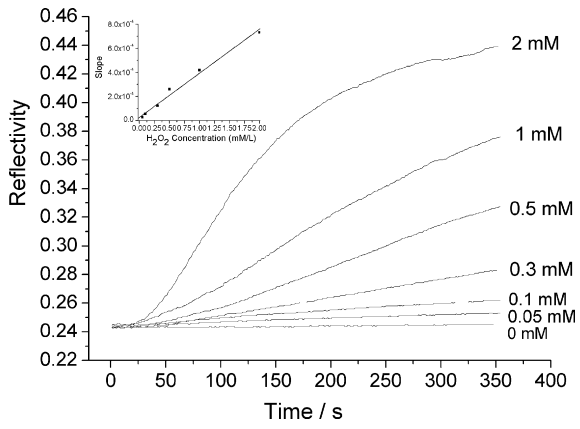

Fig. 5. SPR kinetics curves at various concentration of H_2O_2. The angle of incidence was fixed at 65°. Inset: calibration plot of H_2O_2 concentration with the slope of SPR kinetics curves.

4. Conclusions

We described in this paper an effective method for detecting enzymatic reaction of enzyme with small molecules by electrochemical SPR spectroscopy. This method utilizes the optoelectronic switch of redox polyaniline to amplify the SPR signal. Particularly, we have analyzed the catalytic reaction between H_2O_2 and HRP immobilized in BLM and successfully detected the concentration of H_2O_2 by SPR. These results show the BLM supported by PAn film is a proper substrate for detection of enzymatic reaction by electrochemical SPR method. Furthermore, this electrochemical SPR method provides a potential application for detecting proton transition in BLMs.

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