Layer by layer self-assembly immobilization of glucose oxidase onto chitosan-graft-polyaniline polymers

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Chitosan (CS), an *N*-deacetylated derivative of chitin, is a nature occurring biopolymer found in the exoskeleton of crustaceans, in fungal cell wall, and in other biological materials [1]. It has unusual combination properties [2], which includes excellent membrane-forming ability towards water, good adhesion, nontoxicity, high mechanical strength, especially good biocompatibility and susceptibility to chemical modification due to the presence of reactive amino and hydroxyl function groups. In recent years, chitosan was generally used to immobilize various enzymes. And we have reported the first attempt to prepare chitosan/glucose oxidase (GOD) nanolayered films for electrode modification by the technique of layer-by-layer (LBL) self-assembly [3]. The response current of the CS/GOD LBL films to glucose was improved, as the amount of the immobilization enzyme increased with the increase of the layer numbers to some extent. However, chitosan is nearly nonconductive, it hampers the transition of electron, accordingly, the response current to glucose was not improved too much as expected, especially, the response time gradually extended with the increase of the layer numbers.

Graft copolymerization of polyaniline (PAN) onto chitosan can enhance the conductivity of chitosan film [4]. In recent years, polyaniline is becoming a promising material for potentiometic biosensor [5–7]. It can increase the sensitivity of the biosensor because the proton generation would occur inside the sensitive layer. However, PAN, like many other conductive polymers, is insoluble in common solvents. Moreover, PAN has rather poor mechanical and general physical properties. In general, the composites obtained by incorporating a rigid conducting polymer (such as PAN) into flexible matrix (such as chitosan) can combine the good processability of the matrix and the electrical conductivity of the conductive polymer [8].

In the present study, chitosan film via graft copolymerization with electroactive PAN was investigated. The modified polymer was subsequently functionalized via layer-by-layer electrostatic self-assembly immobilization of GOD. Previous work [4] revealed that surface graft copolymerization of chitosan film with electroactive PAN readily increased the conductivity as well as remained the hydrophilicity and the biocompatibility. The multilayer membrane is expected to have a rapid response and a better sensitivity simultaneous with a higher response current as the layer numbers increase.

Chitosan (Mw = 700,000) and Glucose oxidase (EC1.1.3.4, 340 unit/mg) was purchased from Sigma. Glucose was obtained from Sigma Chem. Co. All other chemicals were analytical grade. The polymerization to synthesis chitosan grafted with polyaniline was carried out as follows. In a typical reaction, 0.2 g chitosan in 40 ml of 2 wt.% acetic acid was combined with 40 ml 1 M HCl containing 1 g aniline at 25 °C. The solution was cooled to 5 °C in an ice/water bath and stirred continuously. Then the 10 ml of HCl solution containing 0.1 g $(NH_4)_2S_2O_8$ was added in it. The solution was kept at 5 °C for 1 hr, then the bath was removed and stirring was continued for 3 hr. To achieve a pH above 7.0, the solution was added 1 M NaOH. The mixture was centrifuged at 10,000 rpm for 0.5 hr. The supernatant was discarded, and the precipitate was washed with deionized H_2O and centrifuged three times. The washed precipitate was dissolved in 2 wt.% acetic acid. The above solution was neutralized by addition of 1 M NaOH solution, and centrifuged to separate. At last, the precipitate was dried to achieve the product.

The preparation of the enzyme electrode was as followed. CS-g-PAN was dissolved in 2% HAc to make 4 mg/ml cationic dipping solution and the glucose oxidase was anionic dipping solution. The surface of the Pt electrode was polished thoroughly with an alumina power and rinsed with distilled water before use. The process of enzyme immobilization was carried out in a typical self-assembly way: the Pt electrode was immersed in

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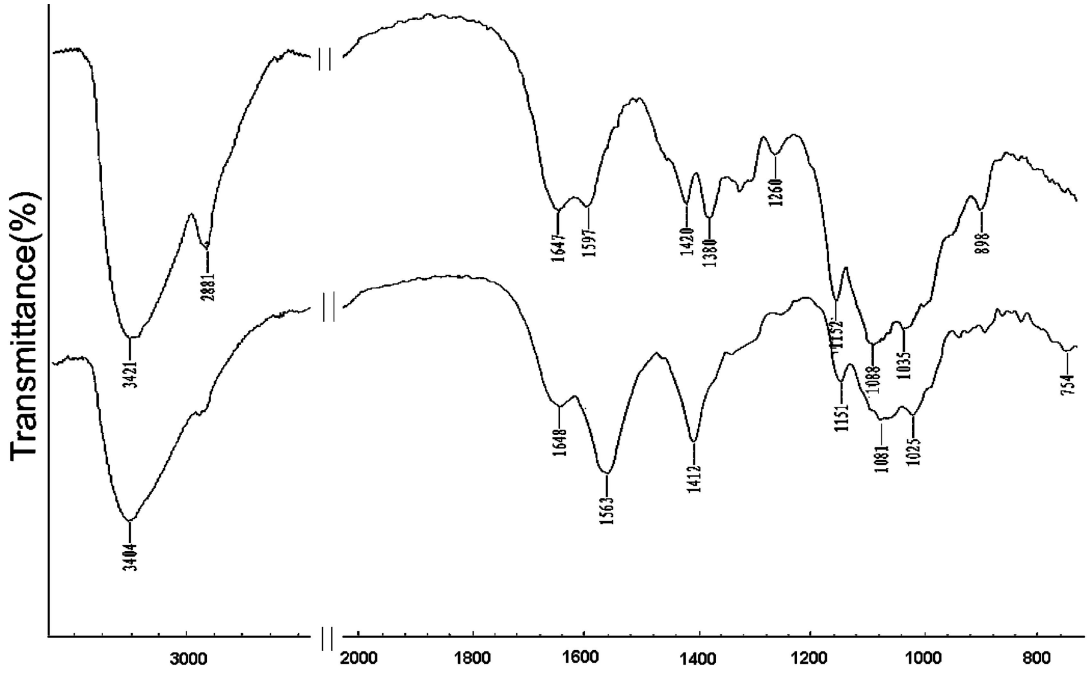

Figure 1 Infrared spectrum of (a) chitosan and (b) grafted chitosan.

either electrolyte solution for 20 min interrupted with 5 min washing with double distilled water. The layer-by-layer films of negatively charged GOD and positively charged CS-g-PAN, denoted $(CS\text{-}g\text{-}PAN/GOD)_n$, where n was the number of bilayer in the films. The resulting enzyme electrode was washed thoroughly with 0.1 M phosphate buffer solution(PBS), and then stored in PBS with pH 6.8 at 4 °C. The layer-by-layer deposition of CS-g-PAN and GOD was also proved by QCM as we have reported in our previous work [3].

The determination principle of the current response is based on the formation of hydrogen peroxide during the enzyme-catalyzed reaction. The hydrogen peroxide is detected by amperometric method. The electrolysis cell consisted of a CS-g-PAN/GOD coated Pt working electrode, a Pt counter electrode and Ag/AgCl as a reference electrode. The electrochemical process was carried out in 0.1 M PBS (pH 6.8) at a constant potential of 0.60 V. The apparatus used for determining the current response was Potentiostat/Galvanostat (Model 283, EG&G PARC with software M270).

FT-IR spectra were used to characterize chitosan grafted with polyaniline. FT-IR spectra were recorded on a BIO-RAD FTS3000 FT-IR spectrometer. Spectra were taken with a resolution of 4 cm^{-1} in the range of 4000–400 cm^{-1}. Samples were thoroughly ground with exhaustively dried KBr and discs were prepared by compression under vacuum.

The infrared spectra of chitosan (a) and grafted chitosan (b) with polyaniline are shown in Fig. 1. From the chitosan spectrum (Fig. 1a), it can be found that the distinctive absorption bands at 3421 cm^{-1}, 2811 cm^{-1} ($-NH_2$ stretching) and 1647 cm^{-1}, 1597 cm^{-1}, 1380 cm^{-1} ($-NH_2$ bending). The absorption bands at 1152 cm^{-1} (anti-symmetric stretching of C–O–C bridge), 1088 cm^{-1}, 1035 cm^{-1} (skeletal vibration involving the C–O stretching) are characteristics of its saccharide structure. The new absorption bands at 3404 cm^{-1}, 754 cm^{-1} ($-NH$ bands) and 1563 cm^{-1}, 1412 cm^{-1} (phenyl bands) can be detected from grafted chitosan IR spectrum (Fig. 1b). In addition, the bends of $-NH_2$ are decreased, which suggests that polyaniline has been successfully grafted onto chitosan backbone.

Amperometric response characteristics of the enzyme electrode to glucose are shown as followed. Fig. 2 shows the effect of the layer number on the biosensor characteristics. Curves a and b show the amperometric current response of the enzyme electrodes modified by CS-PAN/GOD and CS/GOD respectively as a function of layer number. The results showed that the response current of this two kinds of enzyme electrodes was enhanced as the amount of the immobilized GOD increases with

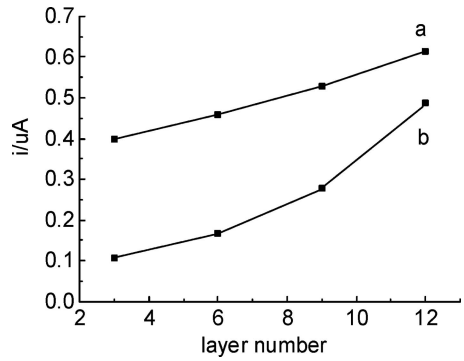

Figure 2 Effect of layer number of self-assembly films on the current response to 5 mM glucose.

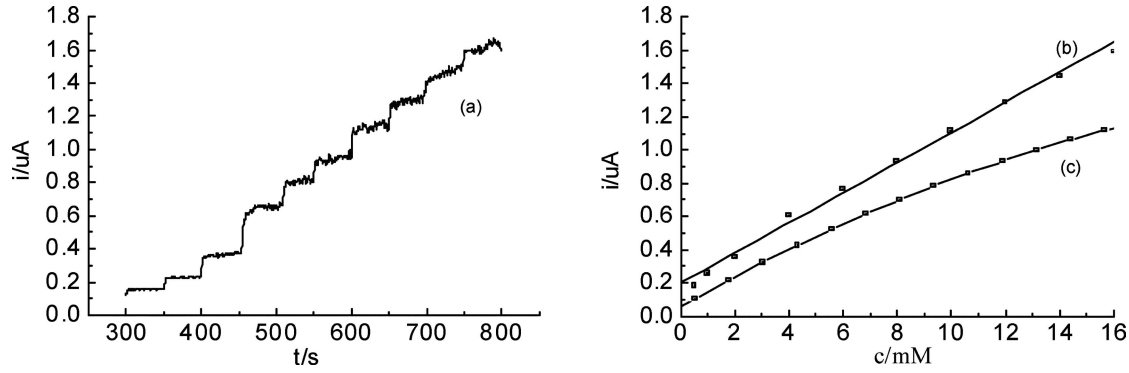

Figure 3 Current response of the $(CS\text{-}g\text{-}PAN/GOD)_{12}$ biosensor in the concentration range of 0.5–16 mM (a), the linear calibration plot of enzyme electrodes modified by $(CS\text{-}g\text{-}PAN/GOx)_{12}$ (b), and $(CS/GOx)_{12}$ (c), respectively.

the increasing of the film's layer number. Whereas, chitosan film via graft copolymerization with electroactive PAN enhances the ability of electron transition. Therefore, compared with CS/GOD, the CS-g-PAN/GOD multilayer films can improve the current response more effectively.

Fig. 3a displays a typical current-time response using the enzyme electrode under the optimal experimental conditions (pH 6.8, working potential 0.6 V) after the addition of successive aliquots of glucose to PBS. A well-defined reduction current proportional to the glucose concentration has been observed. Curves c $[(CS/GOD)_{12}]$ and b $[(CS\text{-}g\text{-}PAN/GOD)_{12}]$ in Fig. 3 show the amperometric current response of the biosensor as a function of glucose concentration. The calibration curve of $[(CS\text{-}g\text{-}PAN/GOD)_{12}]$ is linear with glucose concentration from 0.5 to 16 mM while $[(CS/GOD)_{12}]$ is from 0.5 to 8 mM. The $(CS\text{-}g\text{-}PAN/GOD)_{12}$ biosensor has a broader linear range and higher response current than $(CS/GOD)_{12}$ biosensor, which can satisfy the detection of glucose in human body.

Fig. 4 illustrates the steady-state current response of the biosensor with CS-g-PAN/GOD (a) to 5 mM glucose having a fast response time (5.2 s) while the biosensor with CS/GOD (b) has a slower response time (10.1 s) under the same condition. At the same time, the former has a greater response current. Due to the conductivity of PAN, CS-g-PAN film has a faster transition of electron than CS film. Accordingly, it can enhance the output current and reduce the time of response.

In conclusion, we introduced a new type of amperometric GOD biosensor based on CS-g-PAN/GOD, which was prepared by the technique of layer-by-layer electrostatic self-assembly. The glucose biosensors fabricated using the CS-g-PAN/GOD multilayer film exhibited a rapid response and a higher output current to glucose in the normal and diabetic level.

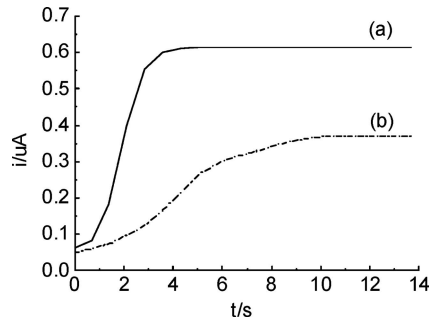

Figure 4 The steady-state current response of GOD biosensor covered with $(CS\text{-}g\text{-}PAN/GOD)_{12}$ (a) and $(CS/GOD)_{12}$ (b) to 5 mM glucose.

Acknowledgments

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