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Biomaterials 27 (2006) 1167–1174
Biomaterials
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A conductive ormosil encapsulated with ferrocene conjugate and multiwall carbon nanotubes for biosensing application

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Received 23 April 2005; accepted 11 July 2005
Available online 24 August 2005

Abstract

Highly non-toxic and conductive ormosil composite film was prepared using (3-aminopropyl)triethoxysilane and 2-(3,4-epoxycyclohexyl)-ethyltrimethoxysilane by doping with ferrocenemonocarboxylic acid–bovine serum albumin (FMC–BSA) conjugate and multiwall carbon nanotubes (MWNTs). With glucose oxidase (GOD) as a model enzyme this film could be used to design an amperometric biosensor for glucose determination. The entrapped FMC–BSA conjugate performed excellent redox electrochemistry and the immobilized GOD was highly stable. Under optimal conditions this biosensor was able to detect glucose with a detection limit of 20 μM ($S/N = 3$) in the linear range of 0.05–20.0 mM in flow system, which was wider than the batch amperometric mode, with an analysis time of 25 s for each sample. The value of K_M^{app} was 6.6 mM. The proximity of these three components FMC–BSA, MWNTs and GOD enhanced the electron transfer between the film and electrode. This film could be used efficiently for the entrapment of other redox bioactive compounds and biosensing/bioelectrochemical applications.

Keywords: Biosensors; Flow analysis; Composite; Multiwall carbon nanotubes; Ferrocenemonocarcoxylic acid–bovine serum albumin conjugate; Glucose oxidase

1. Introduction

Since last two decades considerable attention has been paid in development of new non-toxic and highly conductive materials for biosensing applications and design of diagnostic kits and tools. Binding of bioactive molecules on transducer and electron transfer capacity of supporting film greatly influence the biosensor characteristics. In recent years sol–gel chemistry has paved a versatile path for the immobilization of biomolecules with acceptable stability and good activity retention capacity [1,2]. Considerable work has been done on immobilization of enzymes in inorganic sol–gels like tetramethoxysilane (TMOS) and tetraethoxysilnae (TEOS) doped with other dopants [2,3]. A novel organically modified sol–gel (ormosil) has been introduced for the improvement of sol–gel characteristics such as porosity, hydrophilicity and hydrophobicity [4]. The resulted ormosil matrices retain excellent properties such as controllable porosity, reactive functional groups, low temperature condensation and high binding capacity with electrode surfaces [2,4]. Several enzymes such as horseradish peroxidase (HRP), glucose oxidase (GOD) and acetylcholinesterase have been successfully immobilized into to ormosil matrices and employed in sensing applications [5–7]. However, electron transfer efficiency of redox enzymes is poor in absence of mediator, because enzyme active sites are deeply embedded inside the protein. The sensitivity of resulted biosensors can be significantly improved by the addition of mediators in solution [5,8] or in the matrices [9]. For the improvement of conductivity of sol–gel matrices carbon nanotubes (single and multiwall) or palladium have also been incorporated in the matrices [10,11]. This work incorporated both carbon nanotubes and

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doi:10.1016/j.biomaterials.2005.07.018

mediator in conjugate form into enzyme-modified ormosil matrix to produce a composite film for preparation of amperometric biosensor.

Leakage is a main problem in the entrapment of mediator (low molecular weight) compounds in sol–gel matrices. This problem can be resolved by covalent linking of mediator with ormosil/sol–gel precursors or with high molecular weight compounds before immobilization. Some biosensors have been reported through direct linking ferrocene with sol–gel precursors [12] and poly(oxyethylene) chain [13]. Another often-used approach for the immobilization of mediators on electrode surface is direct conjugation of mediator with active [14–16] and inert proteins [17–19] before fixed on the surface. The direct conjugation of mediator on active biomolecules is highly advantageous but some limitations also associated. During the conjugation active biomolecules might lose catalytic activity. Whereas the covalent linking of mediator with inert proteins is comparatively convenient and high number of redox molecules can be immobilized on protein surface. Here bovine serum albumin (BSA), an inert protein with a 35–40 reactive amino groups (lysine), was used to link ferrocene monocarboxylic acid (FMC) to form FMC–BSA conjugate for immobilization of mediator. The conjugate could be easily entrapped in sol–gel/ormosil films without leaching. Compared with active proteins BSA is cheap and can immobilize a great number of redox molecules by that good electron mediation and high signal can be generated.

Carbon nanotubes have attracted much attention during the past decade due to their unique mechanical, chemical, and electrical properties [11,20]. The incorporation of carbon nanotubes into ormosil matrices can improve the conductivity of the resulted film and facilitate the electron transfer among immobilized enzyme, mediator, and electrode. In view of the advantageous features of ormosil, multiwall carbon nanotubes (MWNTs) and ferrocenemonocarboxylic acid-BSA (FMC–BSA) conjugate, this work prepared a highly non-toxic and conductive ormosil composite film. To prove the functionality and biosensor application of the new ormosil composite, GOD was taken as a model enzyme. The present biosensor exhibited good analytical performances such as sensitivity, precision, accuracy, analytical time, stability and reproducibility towards the quantification of glucose.

2. Materials and methods

2.1. Material

GOD (EC 1.1.3.4, Type X.S from *Aspergillus niger*), β-D(+)-glucose, *N*-2-hydroxyethylpiperazine-*N*′-(2-ethanesulfonic acid) (HEPES), polyethylene glycol (PEG) and *N*′-hydroxysuccinimide (NHS) were purchased from Sigma, St. Louis, USA. (3-aminopropyl)triethoxysilane (APTES) and 2-(3,4 epoxycyclohexyl)-ethyltrimethoxysilane (Epoxy) were obtained from Fluka Chemie, GmbH. BSA, FMC and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimidhydrochlorid (EDC) were purchased from Merck-Schuchardt, Hohenbrunn bei München and used as received. Multiwall carbon nanotubes (MWNTs, with length of 1–2 μm, external diameter of 10–20 nm and surface area of 40–300 m^2/g) were procured from Shenzhen Nanotech Port Co. (China). Glassy carbon electrodes (GCE, 3.0 mm diameter) were obtained from CH Instruments, Inc. (USA).

2.2. Preparation of FMC–BSA conjugate

FMC–BSA conjugate was prepared through carbodiimide coupling, as shown in Fig. 1 [16,17]. In brief, after 2.76 mg of FMC was dissolved thoroughly in 700 μl of 50 mM pH 9.3 HEPES buffer, the pH of resulted solution was adjusted to 7.3 (±0.1) with 3.0 M HCl. Eleven milligram of NHS and 15.0 mg of EDC were dissolved in the solution followed with continuous stirring for 45 min. Solution of 2.64 mg BSA dissolved in 300 μl of 50 mM pH 7.3 HEPES was then added drop by drop to the solution under continuous stirring at a low stirring rate and leaved at room temperature for 20 h. The molar ratio of BSA to FMC was 1:30. After completion of the incubation, the produced conjugate was centrifuged for 10 min at 5000 rpm to remove the precipitates. Finally, the obtained conjugate was dialyzed in a dialysis bag against 0.1 M pH 7.3 phosphate buffer (PBS) at room temperature for 24 h by changing the buffer every 6 h to remove non-conjugated FMC.

2.3. Preparation of FMC–BSA conjugate/MWNTs/ormosil composite

MWNTs were sonicated in water for 20 min and ethanol for another 30 min, and dried at 80 °C for 30 min. 25.0 mg/ml of MWNTs suspension was first prepared. 25.0 μl solution of PEG (4.0% w/v), 17.0 μl APTES, 9.0 μl Epoxy, 10.0 μl solution of FMC–BSA conjugate (2.64 mg protein/ml), 4.0 μl MWNTs suspension (25.0 mg/ml) were added sequentially to 125.0 μl water and mixed thoroughly on stirrer. After 2 min 2.0 μl of

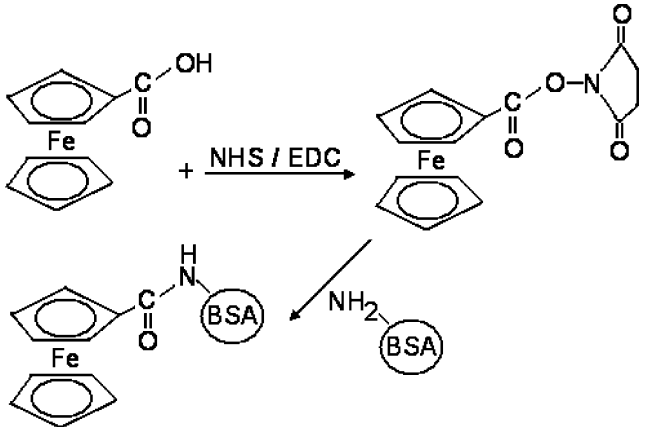

Fig. 1. Schematic diagram of the FMC–BSA carbodiimide coupling mechanism.

0.1 M HCl was added to the mixture and stirred again for 30 s. Immediately, 3.0 μl of the obtained mixture was cast on GCE and allowed for the condensation at room temperature for 3 h and at 4 °C for 24 h to form a FMC–BSA conjugate/MWNTs/ormosil composite film. For the preparation of enzyme-incorporated composite film 10.0 μl GOD (4698.0 IU/ml in pH 7.0 PBS) was added to the mixture before casting. After the composite film modified electrode was washed thoroughly with 0.1 M pH 7.0 PBS it was stored at 4 °C till use.

Prior to modification, the GCE was polished with 1.0, 0.3 and 0.05 mm alumina slurry (Beuhler), respectively, and rinsed thoroughly with doubly distilled water between each polishing step. The electrodes were then successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and then allowed to dry at room temperature.

2.4. Electrochemical studies

Electrochemical measurements were performed on a BAS-100B electrochemical analyzer (Bioanalytical Systems Inc., USA) with a three-electrode system. The composite film modified electrode, platinum wire and saturated calomel electrode (SCE) were used as working, auxiliary and reference electrodes. Amperometric experiments were carried out in a stirred system by applying a potential step of 300 mV to the working electrode. Aliquot of glucose standard solution was added to 0.1 M pH 7.0 PBS after steady state current was achieved, then current-time data were recorded.

2.5. Apparatus

Scanning electron micrographs of ormosil composites were obtained with a Hitachi X-650 scanning electron microscope (Hitachi Lid, Tokyo, Japan). UV–vis absorbance spectroscopy was carried out using a UV-2401 spectrophotometer (Shimadzu; Japan).

3. Results

3.1. Characteristics of FMC– BSA conjugate

The absorption spectra of BSA, FMC and FMC–BSA in the range of 600–200 nm are shown in Fig. 2. As generally proteins BSA shows a maximum absorption at 280 nm (curve a), which shifts to 260 nm after reaction with FMC (curve b). At the same time the reaction also leads to a decrease of the peak occurring at 225 nm, while FMC does not show any absorption (curve c). These phenomena may be due to the linking of FMC to BSA surface or the formation of BSA and FMC aggregates.

As shown in Fig. 3, the FMC–BSA conjugate-entrapped ormosil film modified electrode exhibited a pair of stable and well-defined redox peaks at 165 and 235 mV at 100 mV/s (curve b), which were attributed to the redox of ferrocene group in the FMC–BSA conjugate. When the FMC–BSA conjugate entrapped ormosil film was formed on a MWNTs modified electrode the cyclic voltammogram showed a greater background with a slight increase in the redox peak currents (curve c). However, in comparison of curve d with curve c FMC–BSA conjugate/MWNTs/ormosil composite film modified electrode displayed a lower background current, the redox peak currents of the immobilized ferrocene group increased for about 90%, and the peak separation also slightly decreased. The further increases of the redox peak currents were due to the closer proximity between MWNTs and conjugated

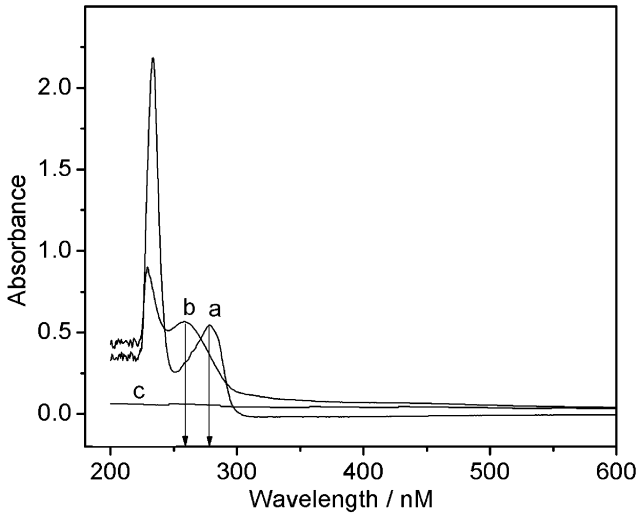

Fig. 2. Spectra of (a) 0.02 mM BSA, (b) 0.02 mM FMC–BSA conjugate and (c) FMC in solutions.

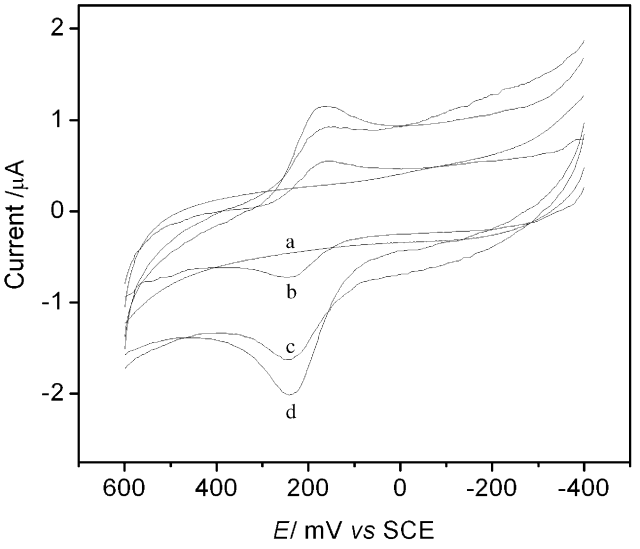

Fig. 3. Cyclic voltammograms of (a) ormosil film, (b) FMC–BSA conjugate/ormosil film, (c) MWNTs covered by FMC–BSA conjugate/ormosil film and (d) FMC–BSA conjugate/MWNTs/ormosil composite film modified electrodes in 0.1 M pH 7.4 PBS at 100 mV/s.

mediator in the composite film. The presence of MWNTs in the composite improved the conductive property of ormosil matrix.

With an increasing scan rate the peak currents increased, and the peak separation also increased slightly (Fig. 4). The plot of peak current vs. square root of scan rate from 10 to 250 mV/s showed a linear relation (inset in Fig. 4), indicating a non-surface-controlled electrode process.

Fig. 5 shows SEM image of ormosil film and distribution of both MWNTS and the FMC–BSA conjugate in the ormosil thin film. The ormosil film is very smooth and crack free (Fig. 5a). MWNTS and FMC–BSA conjugate are homogeneously distributed in the ormosil matrix without any aggregation or clump (Fig. 5b and c). This homogeneous distribution of MWNTs and FMC–BSA conjugate provides close interactions with each other and improves the conductivity of the film. Furthermore, such homogeneous distribution of dopants will help in close interactions among enzyme, mediator conjugate and MWNTs though the fast electron shuttle occurs.

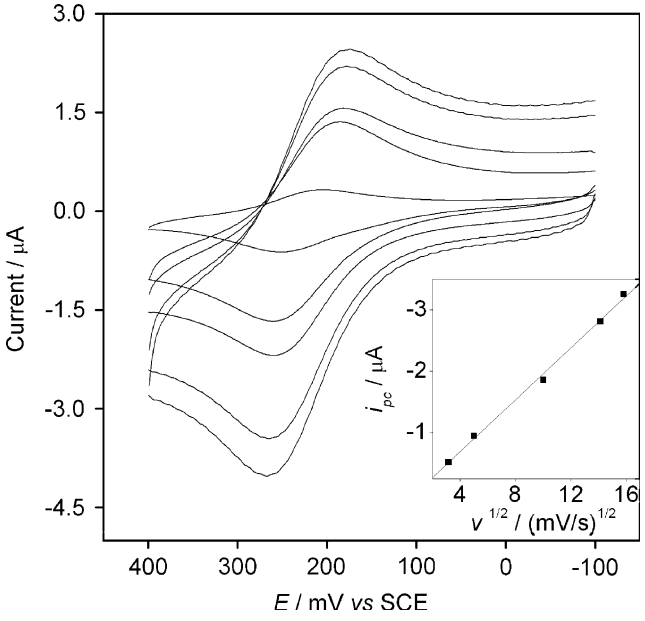

Fig. 4. Cyclic voltammograms of FMC–BSA conjugate/MWNTs/ormosil composite film modified electrode in 0.1 M pH 7.4 PBS at 10, 50, 100, 200 and 250 mV/s (from internal to external). Inset: plot of anodic peak current vs. $v^{1/2}$.

3.2. Influence of applied potential and pH on biosensor response

Upon the incorporation of GOD in the composite film the obtained modified electrode showed a response to glucose (Fig. 6). After addition of 15 mM glucose to PBS the cyclic voltammogram of the GOD incorporated FMC–BSA conjugate/MWNTs/ormosil composite film displayed an electrocatalytic response. The anodic peak current increased and the cathodic current decreased.

The influence of applied potential on the amperometric response of the biosensor to glucose was shown in Fig. 7. The steady-state current quickly increased with the increasing positively applied potential from +50 to +400 mV and reached a maximum response at +300 mV. The applied potential of +300 mV was close to the anodic peak potential of cyclic voltammogram at 100 mV/s (Fig. 6). This work used +300 mV as the applied potential for following amperometric measurements. The bioactivity of the native or immobilized GOD depends on the solution pH. The optimal pH reported for GOD is usually in the range of 6.5–7.5 [3,5,8], which varies with immobilization method and microenvironment around the enzyme. Thus the effect of pH was examined in the range of pH 5.5 to 8.0. This biosensor showed a maximum response at pH 7.0 (inset in Fig. 7).

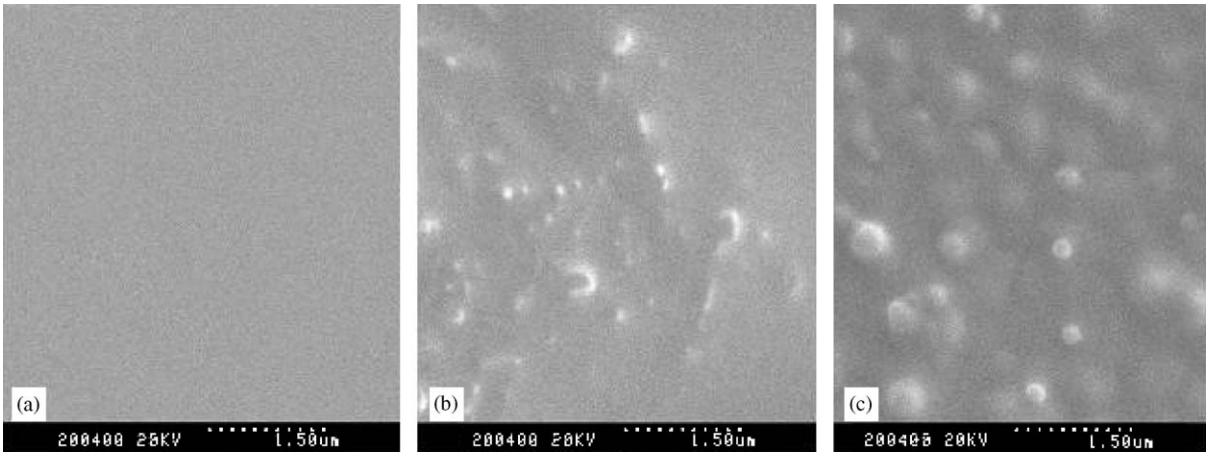

Fig. 5. Scanning electron microscopy of (a) ormosil film, (b) MWNTs/ormosil composite film (c) FMC–BSA conjugate/ormosil film.

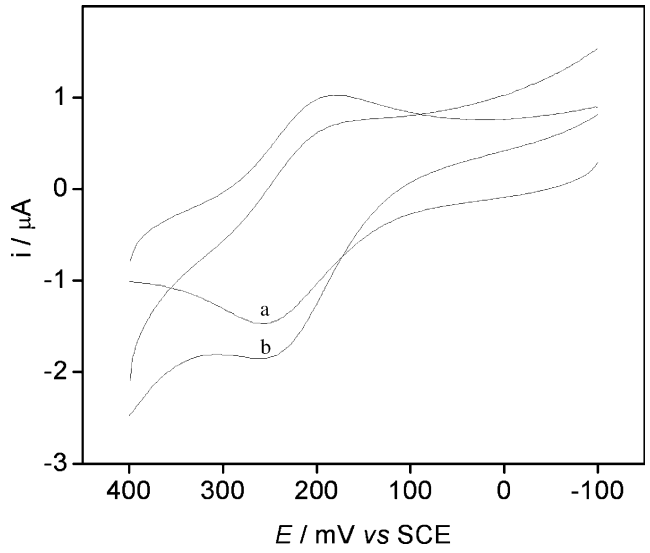

Fig. 6. Cyclic voltammograms of GOD entrapped in FMC–BSA conjugate/MWNTs/ormosil composite film in (a) absence and (b) presence of 15 mM glucose in pH 7.0 PBS at 100 mV/s.

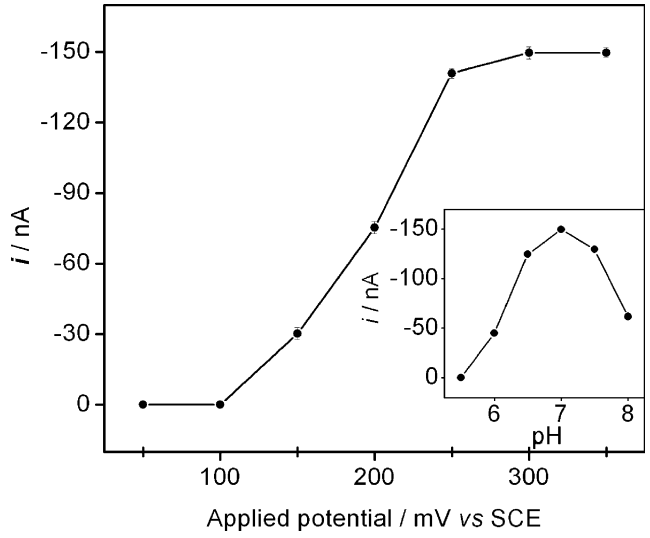

Fig. 7. Effect of applied potential on amperometric response of biosensor in 0.1 M pH 7.0 PBS containing 5.0 mM glucose. Inset: effect of buffer pH on amperometric response of 5.0 mM glucose at +300 mV.

3.3. Condition optimization for sensor preparation

In order to prove the applicability of the proposed ormosil composite glucose biosensor the fabrication parameters were firstly optimized. As shown in Fig. 8 with increasing of enzyme level in the film the catalytic response increased and reached a maximum value at 0.7 IU (700 mIU per electrode). When the amount of the enzyme further increased from 0.7 to 2.5 IU the response decreased slowly by 5%, which might be due to the fact that the increased protein concentration decreased the conductive property of the composite film. Similarly,

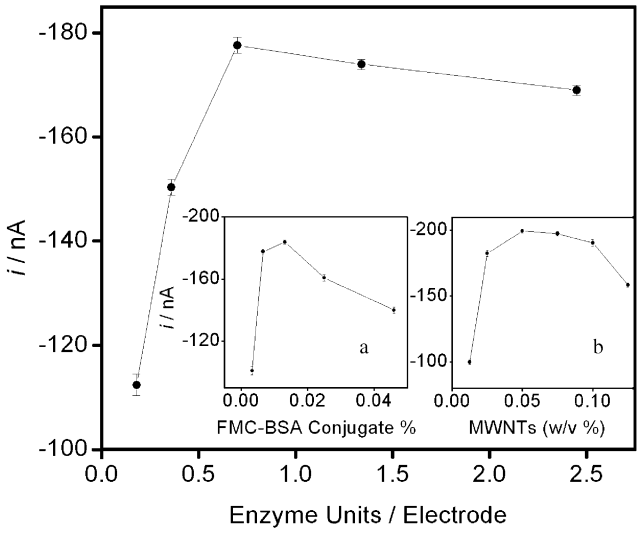

Fig. 8. Influence of enzyme amount on electrode surface on amperometric response of biosensor in 0.1 M pH 7.0 PBS containing 5.0 mM glucose at +300 mV. Inset: effects of (a) FMC–BSA conjugate level and (b) MWNTs amount in FMC–BSA conjugate/MWNTs/ormosil composite film on biosensor response.

with the increasing conjugate level in ormosil composite the steady-state current increased. After the conjugate level of 0.013% (%w/v protein) the response gradually decreased (inset a in Fig. 8a). Although MWNTs could improve the conductive property of the composite film and the amperometric response increased quickly with the increasing amount of MWNTs in the ormosil film, a gradually decreased response occurred at the MWNTs amounts more than 0.05% (w/v) (inset b in Fig. 8). The similar appearance was also reported for determination of dopamine and ascorbic acid in [20]. Hence 0.7 IU GOD, 0.013% (%w/v protein) FMC–BSA conjugate and 0.05% (w/v) MWNTs were selected for preparation of the glucose biosensor.

3.4. Calibration curve for glucose

The calibration curve for glucose detection was shown in Fig. 9. As shown in inset a of Fig. 9, upon successive addition of glucose to PBS the amperometric response at +300 mV increased. The biosensor achieved 95% of the steady-state current in 15 s after addition of glucose. The response was linear over two wide concentration ranges from 0.06 to 8.0 mM and 9.0 to 18.0 mM (insets b and c in Fig. 9) with correlation coefficients of 0.998 and 0.999, respectively. The detection limit was 20.0 μM at a signal to noise of 3. At high glucose concentration the calibration curve showed a Michaelis–Menten type response. The apparent Michaelis–Menten constant $K_M^{app} = 6.6$ mM calculated from the Lineweaver–Burk plot [21] (inset d in Fig. 9). This K_M^{app} value was lower than those of the native GOD in solution (27.0 mM) [22],

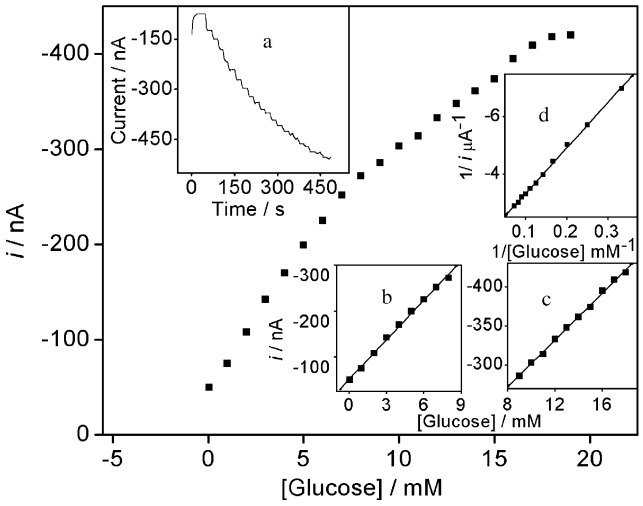

Fig. 9. Amperometric response of biosensor to glucose concentration in 0.1 M pH 7.0 PBS at +300 mV. Inset: (a) typical steady state response curve, (b) and (c) linear plots, and (d) Lineweaver–Burk plot.

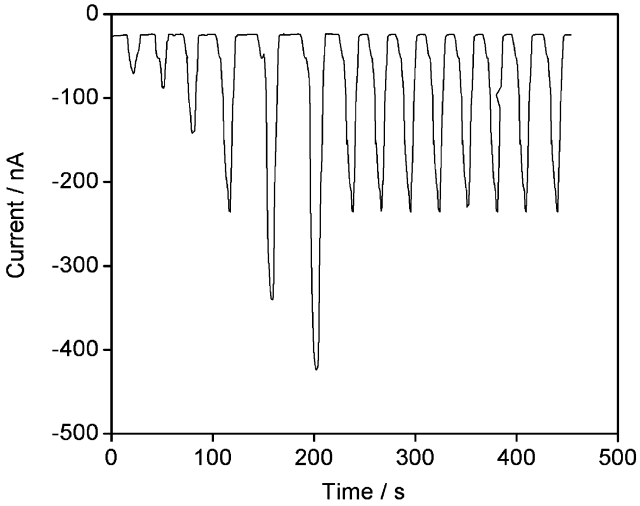

Fig. 10. Flow injection analysis for the glucose concentrations of 0.5, 1.0, 5.0, 10, 15 and 20 mM (from left to right) and 10.0 mM glucose for 8 subsequent additions at +300 mV using 0.1 M pH 7.0 PBS as carrier buffer at 1.0 ml/min.

the immobilized GOD in sol–gel/PVA-*g*-P(4-VP) (20 mM) [23], sol–gel/chitosan composite (21 mM) [3], Pt/PB/GOD-Pan (10.3 mM) [24] and polypyrrole (25.3 mM) [25], and that of 20 mM for GOD bound to self-assemble monolayer electrode [26]. Thus, the GOD encapsulated in FMC–BSA conjugate/MWNTs/ormosil composite film possessed higher affinity to glucose.

3.5. Flow injection analysis

Flow injection detection was carried out to evaluate the possibility of the developed sensor for automation detection. The flow rate used for glucose measurements was an important parameter since the process involved the enzymatic reaction kinetics and the approach of glucose to the immobilized enzyme. An optimal flow rate of 1.0 ml/min was obtained by evaluating the analytical performance of the sensor, the peak width and the measurement reproducibility. Fig. 10 shows a typical response for flow injection detection of glucose at the applied potential of +300 mV. The steady base line could be obtained within 5 s, afterwards the detection of glucose could be performed by subtracting the base value. Flow injection analysis exhibited a wider linear range than that in stirring mode. One linear calibration range from 0.05 to 20 mM ($R = 0.9974$) with a sensitivity of 0.018 μA/mM was obtained. This linear range was also wider than those of 0.07–15 mM [8] and 0.3–20 mM [27] with flow injection analysis. The response time was also very fast. At the flow rate of 1 ml/min this system could detect one sample in 25 s. The reproducibility of the sensor was evaluated by monitoring the response for 8 replicate injections of 10 mM glucose. The relative standard deviation was 1.12% (207.9 ± 2.33 nA), indicating good reproducibility and stability of the biosensor.

3.6. Fabrication reproducibility and storage stability of the sensor

To evaluate the fabrication reproducibility of the biosensor for glucose six biosensors were made independently. The relative standard deviations of the amperometric responses at the glucose concentrations of 1.0 ($n = 3$) and 10.0 mM ($n = 3$) were 2.52% (60.6 ± 1.52 nA) and 1.2% (209.3 ± 2.51 nA), respectively, showing good reproducibility. The biosensor was stable. After stored in dry at 4 °C for 110 days it could retain 96% of initial activity.

3.7. Effect of interferents and real sample analysis

The effect of potential interferents such as uric acid and ascorbic acid on the response of the glucose biosensor was evaluated in 0.1 M pH 7.0 PBS containing 5.0 mM glucose. 0.2 mM uric acid and 0.1 mM ascorbic acid could produce an increase of the response by 2.0% and 4.0%, respectively. The increase was due to the oxidation of the interferents at applied potential of +300 mV. The FIA of glucose in serum samples was performed on the biosensor utilizing a calibration method. The samples were diluted to its half concentration by mixing it with 0.1 M pH 7.0 PBS. Five parallel determinations were carried out for three serum samples. The glucose levels were determined to be 4.5 (±0.15), 9.5 (±0.2) and 13.4 (±0.32) mM, close to 4.2 (±0.1), 9.1 (±0.12) and 12.9 (±0.13) mM obtained using an enzymatic kit (Glucose Trinder kit #315–100;

Sigma Chemical Co, St Louis, MO; USA) based on the work of Trinder [28], showing an acceptable accuracy.

4. Discussion

This study was mainly focused on the development of highly non-toxic and conductive ormosil matrices, which would be useful in biosensing application. The proposed ormosil composite doped with FMC–BSA and multiwall carbon nanotube exhibited excellent electrochemistry of immobilized ferrocene group and conductive properties. The electrochemical behavior of the encapsulated ferrocene was analogous to that observed in solution. The dependence of the cyclic voltammogram on scan rate (Fig. 4) indicated the electron transfer rate was rather fast. The peak current was proportional to the square root of scan rate, which suggested the electrode process involved the gradient of counter ion across the solution and/or the pores of the ormosil composite film to neutralize the charge change during the electrode reaction. The doping of MWNTs improved the conductive properties of the ormosil, thus both the anodic and cathodic peak currents increased (Fig. 3). Furthermore, the formation of composite could improve more efficiently the electrochemical response of the immobilized FMC–BSA conjugate than the adsorption of MWNTs on electrode surface. The composite film showed lower background current than the adsorption layer of MWNTs. Thus the preparation of the FMC–BSA conjugate/MWNTs/ormosil composite was favorable to fabrication of biosensor due to the close proximity between the MWNTs and conjugate during the redox reaction of mediator and fast electron shuttle between mediator and electrode surface via carbon nanotubes.

The spectroscopic characteristics (Fig. 2), the electrochemical behavior of the film (Fig. 3) and the stability of the resulted biosensor indicated the formation of FMC–BSA conjugate, which prevented the leakage of FMC from the matrix. The presence of BSA also improved the biocompatibility of the composite film. The SEM photographs (Fig. 5) clearly showed the homogeneous distribution of carbon nanotubes and the conjugate in the ormosil matrix without any crack. This gelatin protocol was very simple and did not require sonication for the homogenization of these precursors or components.

The GOD entrapped in ormosil exhibited good catalytic activity when adding its substrate into the reaction solution (Fig. 6). The optimal pH occurred around pH 7.0, close to the pH range in native system [3,5,8]. Thus several dopants were efficiently entrapped in the ormosil matrix without any restriction to interaction for electron transfer. Furthermore, the MWNTs improved the conductivity of ormosil matrix and facilitated electron transfer.

The effect of enzyme level loaded on electrode surface on the amperometric response (Fig. 8) was similar to that of GOD entrapped in sol–gel chitosan composite film [3]. When the level was more than 0.7 IU the response decreased gradually. It was due to the decrease in the conductive property of the composite film, which restricted the electron transfer. The dependence of amperometric response on the amounts of both FMC–BSA conjugate and MWNTs showed a similar tendency. The maximum response occurred at 0.013% (w/v protein) of FMC–BSA conjugate and 0.05% (w/v) of MWNTs, respectively (inset in Fig. 8b). Thus these values were selected as optimal conditions.

Under optimal conditions the obtained biosensor showed very good affinity to glucose, which was better than those reported in [3,23–26] and even better than that in solution [22]. Thus the enzyme entrapped in the composite film was favorable to the enzymatic reaction, and the ormosil matrix did not have any diffusional limitation, which led to a good sensitivity and fast amperometric response (Fig. 9). The reproducibility of both intra-assay and inter-assay and the storage stability were within the acceptable range. The linear range for flow injection detection of glucose was rather wide, and the analytical time for each sample (Fig. 10) was also acceptable, which was much shorter than that of 1.6 min using a miniature needle type sensor [29]. These analytical performances were sufficient for clinical application, because the blood glucose concentration in normal human ranges from 4.4 to 6.6 mM [30].

The excellent analytical performance and stability of the biosensor was due to the good biocompatibility and conductivity of the FMC–BSA conjugate/MWNTs/ormosil composite. The FMC–BSA conjugate contained both ferrocene group and BSA protein. It could act as both a mediator and a stabilizing agent. BSA has been used to improve the thermostability of several enzymes [31]. Thus, the entrapped GOD could be stabilized by the BSA. Another factor for the stabilization of the entrapped GOD was the presence of ormosil that contained hydrophobic and hydrophilic monomers, which resulted in a milder environment around the enzyme. Both factors led to good stability of the biosensor up to 110 days.

5. Conclusions

The proposed FMC–BSA conjugate/MWNTs/ormosil composite shows excellent conductivity, and porosity. The formed film is crack free and swelling free. The non-toxic nature and improved conductive properties of the composite film will allow entrapment of different types of biocatalysts and the construction of biosensors. The

resulted reagentless biosensor displays excellent analytical performance and stability. The composite film can efficiently entrap both mediator and the enzyme without leakage from the electrode surface in stirred and flow analysis modes. Such a biosensor would be highly useful for clinical application and easy for the automation of glucose detection.

Acknowledgment

We gratefully acknowledge the financial support of the Distinguished Young Scholar Fund to H.X. Ju (20325518), the National Natural Science Foundation of China (20275017) and the Specialized Research Fund for Excellent Young Teachers from Ministry of Education of China. KVB and VST are highly thankful to the postdoctoral fellowship from Nanjing University.

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