Bioprocess Biosyst Eng (2008) 31:519–526
DOI 10.1007/s00449-007-0190-4

ORIGINAL PAPER

Development of a high analytical performance amperometric glucose biosensor based on glucose oxidase immobilized in a composite matrix: layered double hydroxides/chitosan

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Received: 16 August 2007 / Accepted: 18 December 2007 / Published online: 4 January 2008

Abstract This paper aimed at showing the interest of the composite material based on layered double hydroxides (LDHs) and chitosan (CHT) as suitable host matrix likely to immobilize enzyme onto electrode surface for amperometric biosensing application. This hybrid material combined the advantages of inorganic LDHs and organic biopolymer, CHT. Glucose oxidase (GOD) immobilized in the composite material maintained its activity well as the usage of glutaraldehyde was avoided. The process parameters for the fabrication of the enzyme electrode and various experimental variables such as pH, applied potential and temperature, were explored for optimum analytical performance of the enzyme electrode. The enzyme electrode provided a linear response to glucose over a concentration range of 1×10^{-6} to 3×10^{-3} M with a high sensitivity of 62.6 mA M^{-1} cm^{-2} and a detection limit of 0.1 μM based on the signal-to-noise ratio of 3.

Keywords Composite matrix · Glucose oxidase · Layered double hydroxides · Chitosan · Biosensor

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Introduction

Due to its application in the diagnostic analysis of diabetes, the development of glucose sensor with high analytical performance seems to be of great importance. The challenge in biosensor development is to create the microenvironment for the immobilized biomolecules on the transducer surface that present the full bio-functionality of the biomolecules over a long time [1]. Thus, it is important to select suitable materials as enzyme immobilization matrixes for the design of high-performable glucose biosensors.

Layered double hydroxides (LDHs), have been demonstrated as attractive materials due to their technological importance in catalysis, separation, optics, medical science and nanocomposite materials engineering [2–4]. LDHs display a layered structure built on a stacking of positive layers ($[M_{1-x}^{II}M_x^{III}(OH)_2]^{x+}$ [5]. The electro-neutrality of the system can be achieved by the presence of changeable anions accompanied with water molecules situated in the interlamellar domains. LDHs have been also successfully utilized as the attractive enzyme immobilization matrix in our previous works [6–9]. Their unusual intercalation properties allow enzyme immobilization without covalent binding and provide host matrixes, which exhibit high hydrophilic character [10].

However, these inorganic clay-films may crack when stored dry, leading to serious problems, such as a short lifetime of the biosensors. In some cases it can lead to the removal of the films from the electrode surface. Concerning the swelling property of the inorganic clay, it may limit the practical application of these biosensors in some cases [11, 12]. In addition, glutaraldehyde is generally used as a chemical cross-linking agent to prevent the release of the immobilized enzyme molecules from the clay films.

Glutaraldehyde contains complicated chemical species of documented cytotoxic nature and can cause the denaturation of the immobilized enzyme to some extent [13]. Therefore, it is necessary to overcome the weakness of the pure LDHs-modified biosensors and to improve their analytical performances.

Chitosan (CHT), the principal derivative of chitin, can be found in the exoskeleton of crustaceans, in fungal cell wall, and in other biological materials. It is a copolymer of glucosamine and *N*-acetylglucosamine units, and it displays a rare combination of physicochemical properties that include and excellent film-forming ability, high permeability toward water, good adhesion, and biocompatibility [14, 15]. Therefore, CHT is an interesting structural material and has been studied as a support for the immobilization of enzymes and the construction of amperometric biosensors [16–21].

In this context, we explored a novel composite system based on LDHs and CHT into the design of amperometric glucose biosensor. Glucose oxidase (GOD) was simply entrapped into this novel composite film. This hybrid material might combine the physicochemical attributes of components and improve their features, exhibiting enhanced biocompatibility, mechanical strength and increased adhesion to electrodes.

Experimental

Reagents

Glucose oxidase (GOD) (EC1.1.3.4, Type II, 108 U mg^{-1}) from *Aspergillus niger* was purchased from Amresco. CHT (CHT, MW $\sim 1 \times 10^6$; >90% deacetylation) was obtained from Shanghai reagent company (China). LDHs $Zn_3Al(OH)_8Cl$, were synthesized by co-precipitation method developed by De Roy [5]. Aqueous solution containing $ZnCl_2$ and $AlCl_3$ ($Zn^{II}/Al^{III} = 3$) was slowly introduced under stirring in a reactor containing freshly decarbonated deionized water. The pH was maintained constant at 8.0 by the simultaneous addition of NaOH. The resulting slurry was then left under stirring for 24 h. The precipitate was isolated through centrifuging and washed with decarbonated water several times. It was then dried in air at ambient temperature. The resulting final white product was characterized with XRD and FTIR. All other reagents were of analytical grade and were used as such without further purification. The LDHs colloidal suspension was prepared in boiled-deioned-distilled water. Other aqueous solutions were prepared in deionized water. A measure of 50 mM of stock glucose solution was prepared daily and stored overnight to reach mutarotational equilibrium before use.

Measurements and apparatus

All electrochemical studies were performed with a conventional three-electrode system. A saturated calomel electrode (SCE) and a Pt foil electrode were used as the reference electrode and the counter electrode, respectively. The working electrode was a platinum disk electrode (diameter 2 mm), polished carefully with 0.05 μm alumina particles on silk followed by rinsing with distilled water and dried in air before use. All measurements were carried out in a thermostated cell containing phosphate buffer solution (0.1 M), at 25 °C. The apparatus used for determining the current response was a PC-1 precise potentiostat. Micrographs were obtained with a XL-30E scanning electron microscope (SEM). Sample films were prepared by casting LDHs or CHT or LDHs/CHT solution onto the platinum foil and dried in air. Before SEM measurement, all the samples were coated with an ultra thin layer of gold to minimize charging. Fourier transform infrared (FT-IR) spectra of the samples were measured on a pressed pellet with KBr, employing a Tensor 27 spectrometer.

Preparation of enzyme electrode

A 2.0-wt% CHT solution was prepared by dissolving CHT flakes in 1.0-wt% acetic acid (HAc), and was then diluted into 0.2-wt% solution (about pH 4.0) by adding water. The LDHs colloidal suspension (2 mg ml^{-1}) was prepared by dispersing LDHs in deionized and decarbonated water stirring overnight. GOD was also dissolved in deionized and decarbonated water with concentration of 4 mg ml^{-1}. To prepare the optimized bio-film electrode, 40 μl of LDHs, 40 μl of CHT and 40 μl of GOD were hand-mixed thoroughly. The resulting solution (25 μl) of the well-defined amount of the aqueous mixtures was spread on the surface of the platinum disk electrode. The coating was then dried at 4 C in a refrigerator.

Results and discussion

Morphologies of LDHs and LDHs/CHT composite films

The surface morphologies of LDHs, CHT and LDHs/CHT composite films were examined by SEM imaging. Figure 1a displays the typical morphology of the LDHs, which exhibits loose and hexagonal particles with the diameter of 0.2–0.8 μm, inducing large pores and channels that should enhance the adsorption ability and permeability of this inorganic membrane. Although porous membrane is

desirable, this inorganic film presents sufficiently large pores for the enzyme to leak into the solution by diffusing through it. Hence, in our previous work based on pure LDHs modified biosensor, the cross-linking reagent, glutaraldehyde was generally used to prevent the leakage of the immobilized enzyme [6–9]. The image B is the typical morphology of pure CHT membrane, which is constructed of short irregular fiber structure. LDHs can mix well with CHT aqueous solution to form a homogenous solution, which can be readily immobilized onto the electrode surface and form a film after its being dried. The image shown in Fig. 1c corresponds to the surface morphology of LDHs/CHT composite film. It is important to note the uniform distribution of LDHs/CHT particles. The porous size is effectively reduced in the present of CHT with LDHs. This implies that LDHs/CHT composite film is a suitable matrix for enzyme immobilization without the aid of other cross-linking agent and the enzyme can be physically entrapped within the hybrid matrix.

FTIR analysis of LDHs/CHT/GOD film

The FT-IR spectra of LDHs (a), CHT (b), GOD (c) and LDHs/CHT/GOD (d) are shown in Fig. 2. Spectra (a–d) exhibit similarly strong and broad overlapping bands at about 3,300 cm^{-1}, which are the stretching vibration of N–H (ν_{N-H}) and O–H (ν_{O-H}). The synthesized LDHs are conformed by the low-frequency bands (ν_{M-O} at 843–609 cm^{-1} and δ_{O-M-O} at 416 cm^{-1}) (spectrum a in Fig. 1) [22]. For CHT (spectrum b in Fig. 1), the peaks at 1,569 and 1,375 cm^{-1} can be assigned to the bending vibration of N–H (δ_{N-H}) and C–H (–CH_2, δ_{C-H}). The very strong peak at 1,066 cm^{-1} is the typical stretching vibration of C–O (ν_{C-O}) band. For native GOD (spectrum c in Fig. 1), the adsorption band in the range from 1,800 to 1,000 cm^{-1} are assigned to the characteristic peaks of stretching vibration of carbonyl groups, bending vibration of amide groups, methyl and methylene groups, and sugar rings in GOD. When GOD was immobilized in the LDHs/CHT composite film, the typical absorbance bands attributed to amide I (at 1,630 cm^{-1}) and amide II (at 1,525 cm^{-1}) cannot be distinguished in the spectrum d in Fig. 1. This spectral change cannot be unambiguously assigned because it falls into the highly mixed vibrational range, in which the contributions from different chemical groups overlap extensively. However, the small absorption peaks at 2,950 and 1,336 cm^{-1} can be still observed in spectrum d of Fig. 1, which is assigned to the typical adsorption peaks of native GOD. This indicates that GOD is effectively immobilized in the LDHs/CHT hybrid material. On the other hand, the characteristic absorption band of LDHs changed from 619 to 644 cm^{-1}. This result shows that there may be intermolecular interaction between enzyme and some specific sites of the composite matrix.

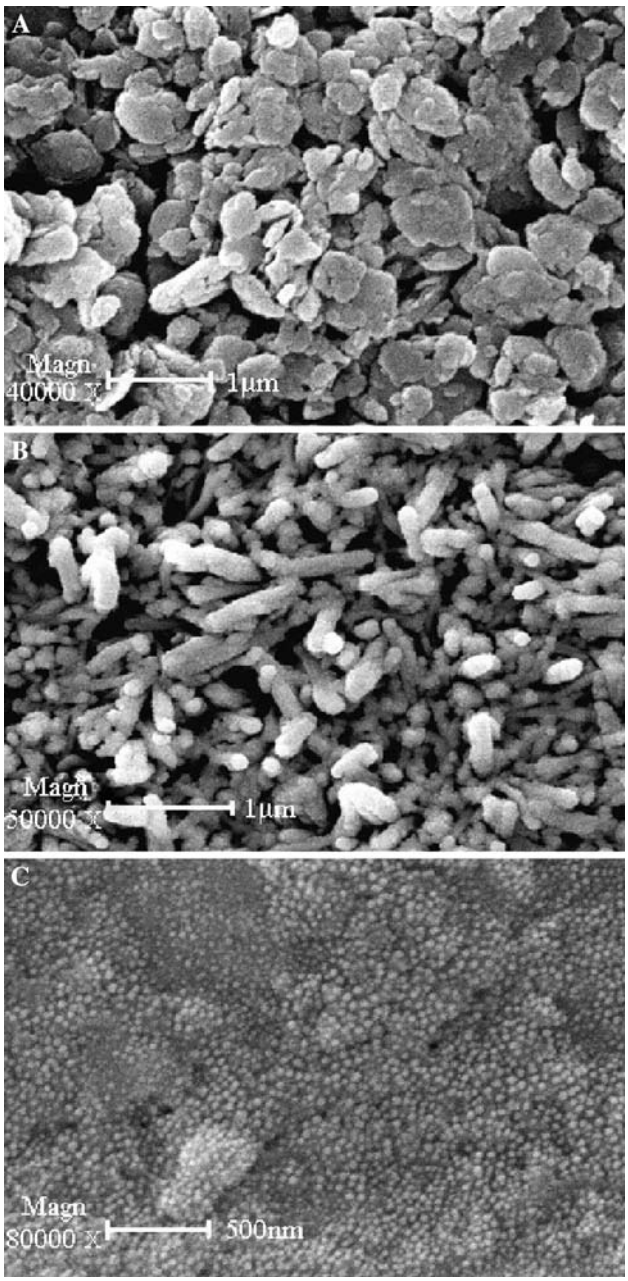

Fig. 1 Scanning electron microphotographs of LDHs (**a**), CHT (**b**) and LDHs/CHT composite (**c**) films

Optimization of conditions of enzyme electrode preparation

In order to optimize the biosensor construction, a systematic study was conducted by varying the composition and the thickness of LDHs/CHT/GOD coating. At a constant amount of GOD (50 μg), the influence of the coating's

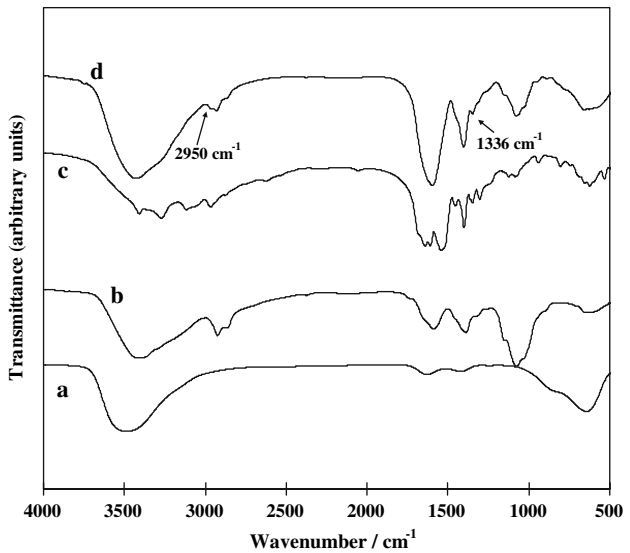

Fig. 2 FTIR spectra of LDHs (*a*), CHT (*b*), GOD (*c*) and LDHs/CHT/GOD (*d*)

composition, which exhibited different LDHs/CHT weight ratios (0.33–4) on the biosensor response to 1 mM glucose, was examined (Curve a in Fig. 3). The best LDHs/CHT weight ratio is found to be 2:1. A slight excess of LDHs in the hybrid film can maintain a hydrophilic microenvironment favorable to the enzymatic process, while with too much LDHs, the biocompatibility of the enzyme matrix decreased, inducing the poor analytical performance of the biosensor.

In our previous work, the optimum ratio between LDHs and GOD was 1 [9]. Thus in this work, the film thickness can be adjusted easily by varying the total amount of

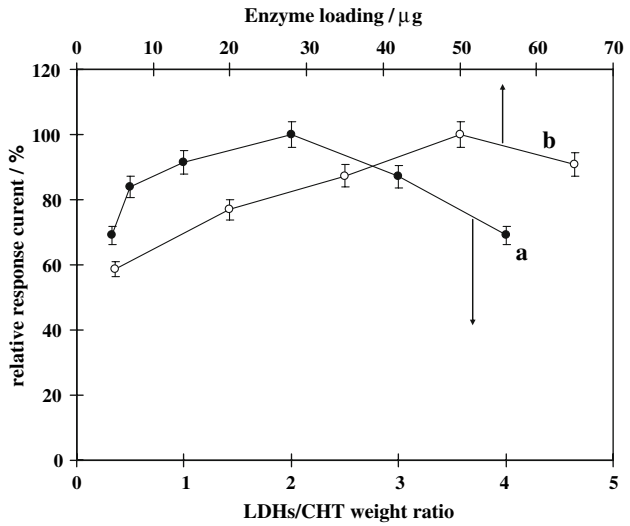

Fig. 3 Influence of LDHs/CHT (w/w) ratio at a constant GOD (50 μg) (*a*) and the biocoating thickness at a constant LDHs/CHT/GOD (w/w/w, 2:1:2) ratio (*b*) on the biosensor response in 0.1 M PBS with pH 6.5, at 25 °C; E_{app} = 0.60 V

LDHs/CHT/GOD (2:1:2) mixture. An initial increase in response to 1 mM glucose is observed upto 50 μg GOD loading at which point the response decreased gradually (Curve b in Fig. 3). An increasing thickness increases the amount of active GOD. When enzyme loading is lower, the response current increases with increment of enzyme loading. This indicates that the catalytic currents are controlled by the enzyme activity in the hybrid film. Then, the biosensor response decreases with the increase in film thickness as previously observed for GOD-biosensors based on polymer films or inorganic coatings as the host matrix [23–26]. Therefore, the configuration based on 50 μg GOD was chosen for the further experiments.

Optimization of measurement variables

The pH value is one of the selected parameters; it affects the response of LDHs/CHT/GOD biosensor to glucose. Figure 4 illustrates the effect of pH on the current response to 1 mM glucose at LDHs/CHT/GOD modified platinum electrode. At the pH values less than 6.5, the response current of the biosensor increases steeply with the increase of pH and then reaches a maximum value at pH 6.5; as the pH over 7.0, the response decreases. The maximum current response is obtained at pH 6.5, which is in agreement with the pH value of 5–6 reported for the native GOD [27, 28]. The output signals of the measurements in pH over 6.5 are lower than that in pH 6.5, which is caused by that the activity of the GOD is poor in alkali [29]. Therefore, PBS at pH 6.5 was selected as the electrolyte in the subsequent experiments.

The dependence of the glucose biosensor response on the applied potential was also evaluated (shown in Fig. 5). The response of the glucose biosensor increases with increasing applied potential from 0.30 to 0.65 V. The

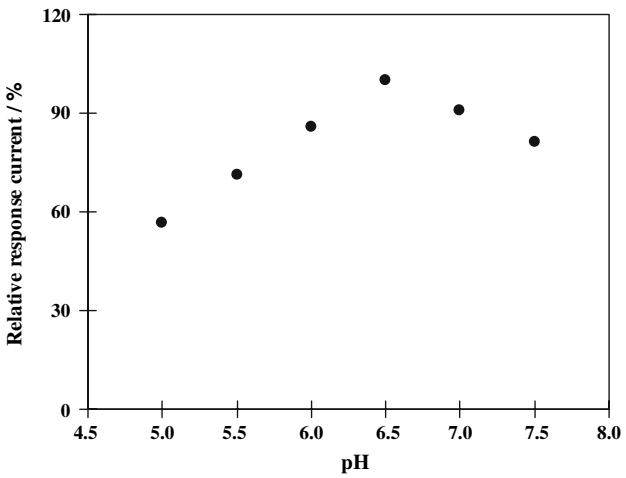

Fig. 4 The effect of pH on the biosensor response to 1 mM glucose

increasing response with applied potential can be attributed to the increased driving force for the electro-oxidation of enzymatically generated H_2O_2. However, an optimum ratio of response-to-background current is observed at 0.6 V. Moreover, the interference of easily oxidizable compounds becomes stronger at higher applied potential. Thus, 0.60 V was selected as the best compromise of applied potential in subsequent experiments.

The effect of temperature on amperometric response of the biosensor was investigated in the range of 5–55 °C (Fig. 6). The response current gradually increases with increasing temperature and reaches a maximum at approximately 50 °C, and then goes down as the temperature rises higher than 50 °C. At higher temperatures, the current response decreases slowly due to the denaturation of the enzyme.

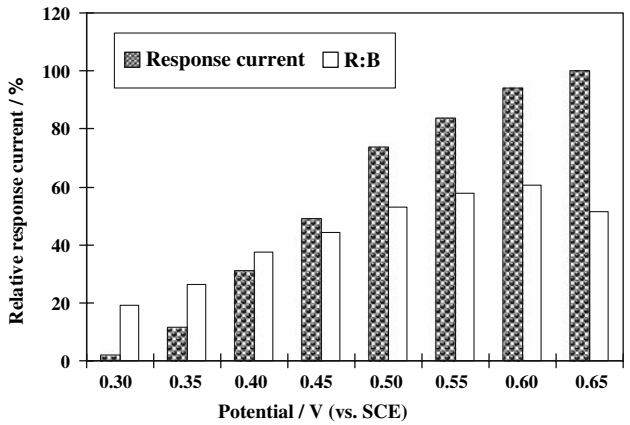

Fig. 5 The effect of applied potential on the biosensor response to 1 mM glucose and the ratio of the response current-to-background current (R:B)

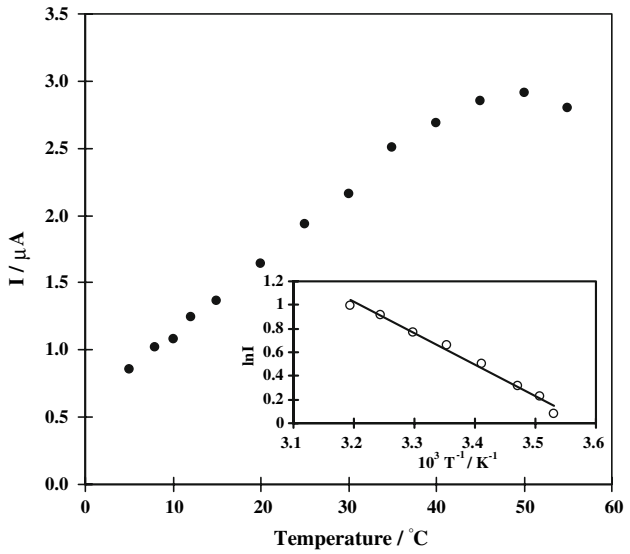

Fig. 6 The influence of temperature on the LDHs/CHT/GOD electrode response to 1 mM glucose with pH 6.5, E_{app} = 0.60 V

The dependence of current on temperature range 10–40 °C can be expressed as an electrochemical version of Arrhenius relationship: $i\ (T) = i_0\ \exp(-E_a/RT)$. The apparent activation energy (E_a) of the enzyme electrode reaction, calculated from the slope of the straight line, is 22.1 kJ mol^{-1} (the standard deviation is 1.9 kJ mol^{-1}), which is similar to that (20.4 kJ mol^{-1}) of the LDHs/GOD electrode [9]. This value is lower than that of GOD/laponite (E_a = 35 kJ mol^{-1}) [6] and is in good accordance with those commonly encountered for immobilized GOD (22.4–50 kJ mol^{-1}). It means that LDHs/CHT provides a friendly microenvironment for GOD. We found that the biosensors exhibited relatively high sensitivity, long-term stability, and operated conveniently at 25 °C, so this temperature was used throughout the experimental work.

Analytical performance of the LDHs/CHT/GOD modified electrode

Under optimal conditions in the determination of the presence of glucose, the response time (defined as the time when 95% of the steady-state current is reached) was less than 5 s (Fig. 7 inset). Such a rapid response indicates a fast mass transfer of the substrate across the film and a fast electron exchange between GOD and its substrates, highlighting the advantages of the LDHs/CHT coating as host matrix for the construction of the biosensor. Figure 7 shows a typical calibration graphs of LDHs/CHT/GOD modified electrode (curve a), LDHs/GOD (curve b) and CHT/GOD (curve c) for the determination of glucose. Among the three GOD

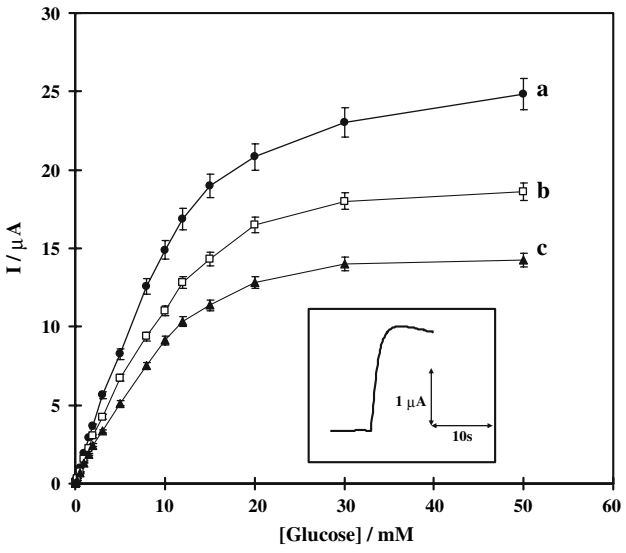

Fig. 7 Calibration curves of LDHs/CHT/GOD (*a*), LDHs/GOD (*b*) and CHT/GOD (*c*) for glucose, in 0.1 M PBS (pH 6.5) at 25 °C, E_{app} = 0.60 V. *Insets* show the amperometric current response of the biosensor to 1 mM glucose

biosensors, LDHs/CHT/GOD exhibits the best analytical performance. The linear range spans the concentration of glucose from 1×10^{-6} to 3×10^{-3} M with a correlation coefficient of 0.9992 ($n = 11$). A low detection limit of 0.1 μM glucose was determined at a signal-to-noise ratio of 3. The glucose sensitivity and maximum current density at saturating glucose conditions (i_{max}) are 62.6 mA cm^{-2} M^{-1} and 1.53 mA cm^{-2}, respectively. This sensitivity for glucose is about 1.7 and 3 times higher than that of GOD biosensor based on pure LDHs (34.8 ± 0.7 mA cm^{-2} M^{-1}) [9] and that of GOD biosensor based on pure CHT (21 mA cm^{-2} M^{-1}) [30], respectively. This analytical performance of the proposed LDHs/CHT/GOD was also compared with those reported in the literature for the GOD-based biosensors using other composite matrixes (Table 1). It appears that the highest sensitivity and the lowest detection limit are obtained with the LDHs/CHT/GOD electrode. The high sensitivity of the LDHs/CHT/GOD sensor may be attributed to the combined merits of LDHs and CHT, such as high biocompatible and hydrophilic microenvironment, and adsorption ability.

The apparent Michaelis–Menten constant, K_M^{app}, can be obtained by the analysis of the slope and the intercept of the plot of the reciprocals of the steady-state current versus glucose concentration (Fig. 8). K_M^{app} is calculated to be 22.2 mM. This value is similar to the reported value for the free enzyme [37], illustrating the nondenaturating character of the enzyme immobilization procedure.

The repeatability of the biosensor fabrication was evaluated via the comparison of the glucose sensitivity of different electrodes. Seven different enzyme electrodes were tested independently for the glucose amperometric response, providing a RSD value of 4.8%. This indicates, in particular, an efficient and repeatable immobilization process of GOD in LDHs/CHT composite matrix although the procedure used was a nonautomatic one.

The main objective of enzyme immobilization on a transducer for analytical purpose is to stabilize the enzyme

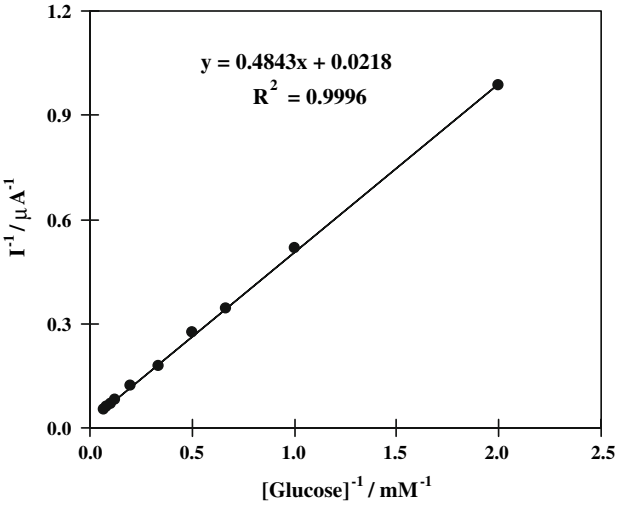

Fig. 8 Lineweaver–Burk plot of I^{-1} versus $[\text{glucose}]^{-1}$ according to data of calibration curve in Fig. 7

for the biosensor to be used repeatedly over a long period. Thus, the operational stability and long-term stability of the LDHs/CHT/GOD electrode were examined. A repeatable current response with a RSD of 2.6% was observed in 150 successive assays of 1 mM glucose. The long-term stability of the LDHs/CHT/GOD electrode stored at 4 °C was investigated by recording periodically its current response to 1 mM glucose. The biosensor retained 90 and 70% of its glucose sensitivity after 12 and 60 days, respectively. The good stability of the biosensor could reflect the inherent stability of immobilized GOD in the inorganic–organic composite matrix. This stability is comparable to our earlier work on the LDHs/GOD biosensor fabricated by glutaraldehyde cross-linking [9]. However, it is still interior to that obtained by pure CHT modified glucose biosensor [30]. Biopolymer CHT in the composite can enhance the biocompatibility, which provides favorable microenvironment for the entrapped enzymes to retain their activity. Moreover,

Table 1 Comparison analytical performance of glucose biosensor based on composite matrix in the literature

Biosensor	Response time (s)	Linear range (M)	Sensitivity		Detection limit (μM)	Reference
			(mA M^{-1} cm^{-2})	(mA M^{-1})		
LDHs/CHT/GOD	<5	1×10^{-6}–3×10^{-3}	62.6	1.87	0.1	This work
CHT/GOD	<8	5×10^{-5}–1.5×10^{-3}	21	–	10	[30]
ZrO_2/CHT/GOD	<10	1.25×10^{-5}–9.5×10^{-3}	–	0.028	10	[31]
Sol-gel/Ferrocene/CHT/GOD	30		3.91	0.274	10	[32]
GOD-GluA-AP-clay	–	1×10^{-5}–3.5×10^{-4}	–	–	1.5	[33]
Poly(4-viny(pyridine-co-styrene)/laponite/GOD	12	1×10^{-5}–1.5×10^{-3}	30	–	–	[34]
GOD-BSA/alumina sol-gel/polyphenol	<10	Up to 4×10^{-2}	1.04	–		[35]
Laponite/poly(pyrrole-pyridinium)	–	$\sim 1.5 \times 10^{-3}$	57	–	–	[36]

Table 2 Influence of easily oxidizable interfering compounds on the response of LDHs/CHT/GOD and LDHs/GOD electrodes

Interfering compound	Physiological concentration (mM)	100 $(i_{G+I} - i_G)/i_G$	
		LDHS/ CHT/GOD	LDHs/ GOD [9]
Ascorbic acid	0.1	3	3
p-Acetamidophenol	0.05	5	5
L-Cysteine	0.02	3	1
Glutathione reduced	2	0	5
Uric acid	0.5	6	13

the adhesive ability of the biomembrane to the electrode surface can be dramatically increased.

The influence of the presence of easily oxidizable endogeneous and exogeneous interferents at their physiological normal levels on the biosensor response to glucose (5.6 mM) was also examined. Their interferences were estimated as a percentage of increase in the biosensor signal recorded in the presence of interferents, compared to the steady-state response to 5.6 mM glucose alone. Table 2 summarized the results of interferences of the LDHs/CHT/GOD and LDHs/GOD. The interfering effect of reduced glutathione and uric acid can be effectively eliminated by the proposed LDHs/CHT/GOD electrode. This is possible due to the less porous structure of the composite film, which does not help the accumulation of the anion in the anionic exchangeable interlayer of LDHs.

Conclusion

Investigations made in this work show the attractive potential offered by the combined advantages of LDHs with CHT. The biopolymer CHT in the composite can not only overcome the brittleness of the pure LDHs film, but also increase the biocompatibility and the adhesive ability of the composite film to the electrode surface, resulting in the improved analytical performance of the biosensor such as the rapid response, high sensitivity, excellent stability and the improved selectivity. Moreover, the usage of glutaraldehyde, which can denature the enzyme, could be avoided. The authors believe that this is a suitable platform for the fabrication of other enzyme biosensors and further work on this is in progress.

Acknowledgments The authors are grateful to the financial supports of National Natural Science Foundation of China (Grant NO. 20505014) and (Grand NO. 20773108), the Key Project of Chinese Ministry of Education (NO. 207041) and Foundation of Jiangsu Provincial Key Program of Physical Chemistry in Yangzhou University.

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