Hybrid Material Based on Chitosan and Layered Double Hydroxides: Characterization and Application to the Design of Amperometric Phenol Biosensor

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A new type of amperometric phenol biosensor based on chitosan/layered double hydroxides organic−inorganic composite film was described. This hybrid material combined the advantages of organic biopolymer, chitosan, and inorganic layered double hydroxides. Polyphenol oxidase (PPO) immobilized in the material maintained its activity well as the usage of glutaraldehyde was avoided. The composite films have been characterized by Fourier transform infrared. The results indicated that PPO retained the essential feature of its native structure in the composite film. The enzyme electrode provided a linear response to catechol over a concentration range of 3.6×10^{-9} to 4×10^{-5} M with a sensitivity of 2750 ± 52 mA M^{-1} cm^{-2} and a detection limit of 0.36 nM based on S/N = 3. The apparent Michaelis−Menten constant (K_M^{app}) for the sensor was found to be 0.13 mM. The activation energy for enzymatic reaction was calculated to be 27.6 kJ mol^{-1}. Furthermore, the biosensor exhibited excellent long-term stability and satisfactory reproducibility.

Introduction

Chitosan, the principal derivative of chitin, can be found in the fungal cell wall and the exoskeletons of lots of arthropods such as crabs and shrimps. It is a biocompatible, biodegradable, and nontoxic natural biopolymer that exhibits excellent film-forming ability and good adhesion.[1,2] Due to its rare combination of physicochemical properties, chitosan is an attractive structural material and has been studied as a support for immobilization of enzymes and the construction of amperometric biosensors.[3−11] It acts as a scaffold for dispersing carbon nanotubes (CNTs) and incorporating firmly CNTs and enzyme at the electrode.[12−17] Hybrid matrix composed of chitosan and ZrO_2 or ZnO combines the advantages of both materials, providing a good electrochemical sensing platform for redox proteins and enzymes.[18,19] A sol−gel matrix in the presence of chitosan can effectively overcome the brittleness of inorganic sol−gel.[20]

Most recently, our group initially introduced this biopolymer into the area of clay-modified biosensor and obtained dramatically enhanced activity and perfect stability of PPO immobilized within a laponite/chitosan nanocomposite matrix.[21] Chitosan aggregated with laponite could enhance biocompatibility, mechanical strength, and adhesion to glass carbon electrodes. In addition, glutaraldehyde, the cross-linking agent, was effectively avoided, which contains complicated chemical species of documented cytotoxic nature and can cause the denaturation of the immobilized enzyme to some extent.[22]

Phenolic compounds are widely used chemicals and released into the environment. They are a class of polluting chemicals, easily absorbed by animals and human through the skin and mucous membranes. Therefore, the determination of phenolic compounds is of great importance due to their toxicity and persistency in the environment. Promising tools largely developed for the detection of phenolic compounds are biosensors based on enzyme PPO immobilization. PPO is a metalloenzyme that contains a binuclear copper active site and catalyzes, in the presence of dioxygen, the hydroxylation of monophenols to catechols (monooxygenase activity), which in turn are oxidized to *o*-quinone (catecholase activity).[23] The phenol biosensor transduction is thus based on the amperometric detection of the enzymatically generated *o*-quinone. Our literature search revealed that until now the most sensitive phenolic biosensor has been fabricated via entrapment of PPO within attractive material: layered double hydroxides.[24,25]

Different from laponite (one of the cationic clays), layered double hydroxides (LDHs) are anionic clays and display a layered structure built on a stacking of positive layers $([M_{1-x}^{II}M_x^{III}(OH)_2]^{x+}$.[26] The electroneutrality of the system can be realized by the presence of changeable anions accompanied by water molecules situated in the interlamellar domains. The positively charged layer may be an attractive point to immobilize biomolecules depending on their isoelectric point. However, the practical application of the above-mentioned anionic clay materials was often limited, unfortunately, by the significant drawbacks, namely, their brittleness, swelling phenomenon, and poor lifetime. Efforts have been made to seek new materials, which could overcome these shortcomings.

In this context, we explore a composite system based on chitosan and LDHs into the design of a phenol biosensor. PPO was simply entrapped into this novel composite film. LDHs can mix well with chitosan aqueous solution to form a homogeneous solution, which can be readily immobilized onto the glassy carbon electrode surface and form a film after it is being dried. The resulting organic/inorganic composite can benefit the merits of the two components and provide a favorable microenvironment for immobilized enzyme.

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Experimental Section

Materials and Solutions. Polyphenol oxidase (PPO) (EC 1.14.18.1) from mushroom (807 U mg^{-1}) was purchased from Amresco. Chitosan (CHT, MW $\sim 1 \times 10^6$; >90% deacetylation) was obtained from Shanghai Reagent Company (China). Layered double hydroxides (LDHs) $Zn_3Al(OH)_8Cl$, denoted [Zn_3–Al−Cl], was synthesized by coprecipitation method developed by De Roy et al.[26] All other reagents were of analytical grade and were used as received without further purification. The LDHs colloidal suspension was prepared in boiled-deionized-distilled water. Other aqueous solutions were prepared in deionized water; phenolic solutions in 0.1 M phosphate buffer solution (PBS) were prepared daily.

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 glassy carbon electrode (diameter 3 mm), which was polished carefully with 0.05 μm alumina particles on silk and was then rinsed with distilled water and dried in air before use. All measurements were carried out in a thermostated cell at 25 °C, containing phosphate buffer solution. The apparatus used for determining the current response was a PC-1 precise potentiostat. Spectrophotometric measurements were carried out with a UV-2550 UV/vis spectrophotometer. Fourier transform infrared (FT-IR) spectra of the samples were measured on a pressed pellet with KBr, employing a Tensor 27 spectrometer. Electrochemical impedance spectra (EIS) measurements were conducted using an Autolab/PG-STAT30 (Eco Chemie, Netherlands) with a three-electrode system. The EIS measurements were performed in 5 mM $Fe(CN)_6^{3-/4-}$ containing 0.1 M KCl. The amplitude of the applied sine wave potential was 5 mV. The impedance measurements were recorded at a bias potential of 180 mV within the frequency range of 0.05 Hz to 10 kHz. Static contact angle measurements were performed at 20 °C with a sessile drop method using an OCA 40 system (German). A droplet of deionized water (2 μL) was gently placed onto the surface. The angle between the edge of the droplet and the surface was measured. Measurements were made in different regions on the surface.

Preparation of Phenol Biosensor. A 2.0 wt % chitosan solution was prepared by dissolving chitosan 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 [Zn_3−Al−Cl] colloidal suspension (2 mg mL^{-1}) was prepared by dispersing LDHs in deionized and decarbonated water with stirring overnight. PPO was also dissolved in deionized and decarbonated water with a concentration of 8 mg mL^{-1}. A defined amount of the aqueous mixtures (for instance, containing 16 μg of [Zn_3−Al−Cl], 4 μg of chitosan, and 16 μg of PPO) was spread on the surface of the glassy carbon electrode. The coating was then dried at 4 °C in a refrigerator. [Zn_3−Al−Cl]/PPO prepared via a cross-linked method by glutaraldehyde[24,25] was used as control.

The amount of enzyme immobilized on the electrode surface was calculated by the difference between the amount of protein initially adsorbed and that detected in washing buffer solution. The latter value was determined by UV spectroscopy following the procedure of Duckworth and Coleman.[27] Catecholase activity of released PPO was measured by the following procedure: 0.04 M potassium ferrocyanide was included in a reaction mixture containing 0.7 mM catechol and unknown PPO samples; its oxidation by enzymatically generated *o*-quinone was monitored spectrophotometrically at 420 nm. The same procedure was repeated with commercial samples of PPO.

Results and Discussion

Characteristics of [Zn_3−Al−Cl], CHT, PPO, and CHT/[Zn_3−Al−Cl]/PPO Film. The amount of PPO truly retained in the film was determined by spectroscopy for both biosensors ([Zn_3−Al−Cl]/PPO cross-linked by glutaraldehyde and CHT/[Zn_3−Al−Cl]/PPO). The amount retained was shown to be 98.6% and 97.8%, respectively. This implies that enzyme can be firmly incorporated within the composite matrix without the aid of other cross-linking agents. Thus, glutaraldehyde was effectively saved.

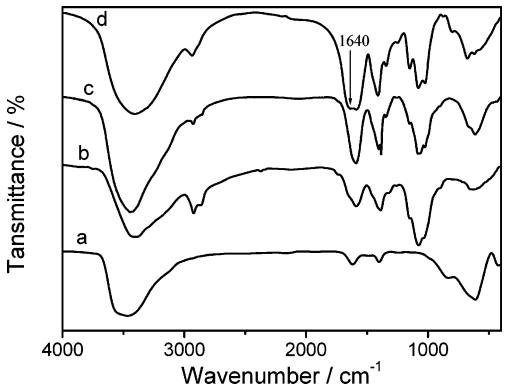

Figure 1. FT-IR spectra of [Zn_3−Al−Cl] (a), CHT (b), CHT/[Zn_3−Al−Cl] (c), and CHT/[Zn_3−Al−Cl]/PPO (d).

The FT-IR spectra of [Zn_3−Al−Cl] (a), CHT (b), CHT/[Zn_3−Al−Cl] (c), and CHT/[Zn_3−Al−Cl]/PPO (d) are shown in Figure 1. Spectra a−d of Figure 1 exhibit similarly strong and broad overlapping bands at about 3300 cm^{-1}, which are the stretching vibrations of N−H (ν_{N-H}) and O−H (ν_{O-H}). The synthesized [Zn_3−Al−Cl] was conformed by the low-frequency bands (ν_{M-O} at 843−609 cm^{-1} and δ_{O-M-O} at 416 cm^{-1}).[28] The peaks at 1587 and 1393 cm^{-1} (Figure 1b,c) can be assigned to the bending vibration of N−H (δ_{N-H}) and C−H (−CH_2, δ_{C-H}). The very strong peaks at 1070 and 1069 cm^{-1} (Figure 1b,c) are the typical stretching vibrations of the C−O (ν_{C-O}) band. The spectrum c is the simple combination of the spectra a and b. This means that there was no interaction between [Zn_3−Al−Cl] and CHT. Positively charged CHT can be just mixed with or be adsorbed on the positively charged [Zn_3−Al−Cl] phase. When PPO was immobilized in the CHT/[Zn_3−Al−Cl] hybrid material, a shoulder peak can be seen at 1640 and 1575 cm^{-1} (spectrum d). The peak at 1640 cm^{-1} is the C═O stretching modes of the amide I band of the protein (PPO). This indicates that PPO is effectively immobilized in the CHT/[Zn_3−Al−Cl] hybrid material. At the same time, the characteristic absorption band of [Zn_3−Al−Cl] changed from 609 to 677 cm^{-1}. This result shows that there might be intermolecular interaction between the enzyme and some specific sites of the composite matrix. Therefore, this CHT/[Zn_3−Al−Cl] film, as a good matrix for enzyme loading, retains the native structure of enzyme.

EIS is a powerful tool for studying the interface properties of surface-modified electrodes. The electron-transfer resistance (R_{CT}) at the electrode surface is an important parameter. Figure 2 displays the Nyquist plots of the impedance spectroscopy of the [Zn_3−Al−Cl], CHT, CHT/[Zn_3−Al−Cl], and CHT/[Zn_3−Al−Cl]/PPO films, respectively. The Nyquist diameter of the electrode deposited with the CHT/[Zn_3−Al−Cl] film (curve c, $R_{CT} \approx 2630\ \Omega$) is smaller than that of [Zn_3−Al−Cl] (curve a, $R_{CT} \approx 3240\ \Omega$), which demonstrates that the hybrid membrane (CHT/[Zn_3−Al−Cl]) allowed greater permeation for the redox probe of $Fe(CN)_6^{3-/4-}$ than the [Zn_3−Al−Cl] film. An obvious increase in the interfacial resistance is observed when PPO was immobilized in CHT/[Zn_3−Al−Cl] film (curve d, $R_{CT} \approx 5620\ \Omega$). The increase in R_{CT} may have been caused by the hindrance of the macromolecular structure of PPO to the electron transfer, and it also confirms the successful immobilization of PPO.

Optimization of Conditions of Enzyme Electrode Preparation. To optimize the biosensor construction, a systematic

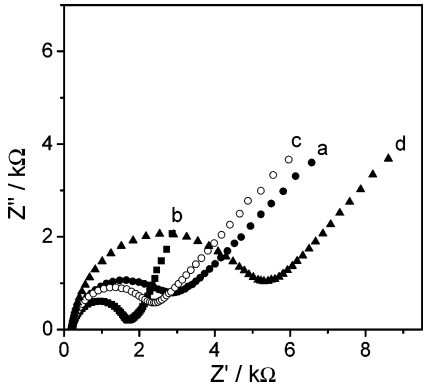

Figure 2. Nyquist plots of the electrochemical impedance spectroscopy (EIS) for [Zn_3−Al−Cl] (a), CHT (b), CHT/[Zn_3−Al−Cl] (c), and CHT/[Zn_3−Al−Cl]/PPO (d) films.

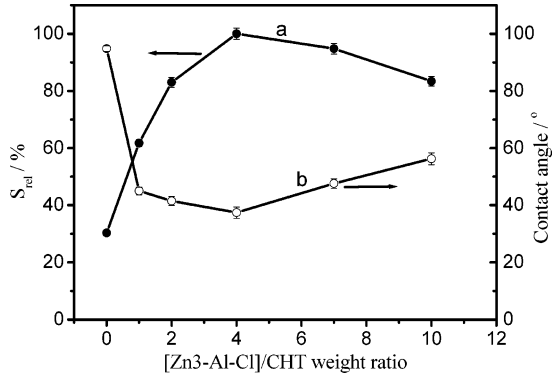

Figure 3. Influence of [Zn_3−Al−Cl]/CHT (w/w) ratio to the biosensor response signal (E_{app} = −0.2 V, in 0.1 M PBS with pH 6.0, at 25 °C) at a constant PPO (16 μg) (a) and to the contact angle of the enzyme immobilization matrix (b).

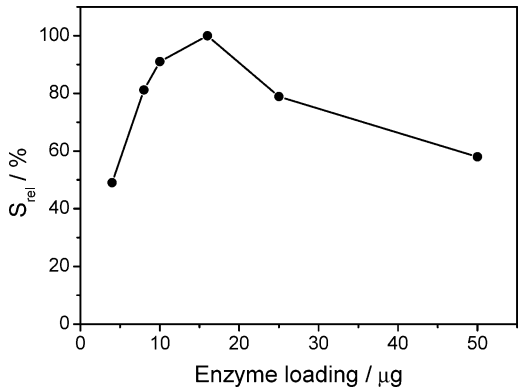

Figure 4. Influence of coating thickness to the biosensor response signal (in 0.1 M PBS with pH 6.0, at 25 °C, E_{app} = −0.2 V) at a constant CHT/[Zn_3−Al−Cl]/PPO (w/w/w, 1:4:4) ratio.

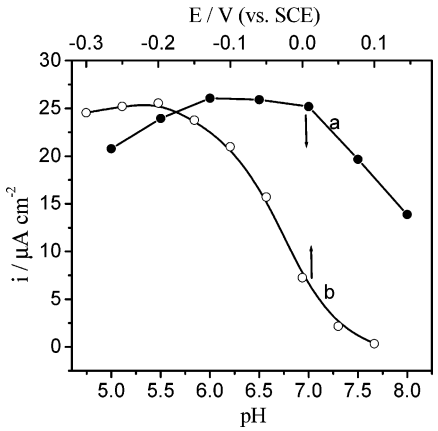

Figure 5. Effect of pH (a) and operating potential (b) on the biosensor response to 10 μM catechol.

study was conducted by varying the composition and the thickness of CHT/[Zn_3−Al−Cl]/PPO coating. Catechol was selected as a model compound to obtain the remarkable biosensor responses. At a constant amount of PPO (16 μg), the influence of the coating's composition, which exhibited different [Zn_3−Al−Cl]/CHT weight ratios (0∼10) on the biosensor response to 10 μM catechol, was examined (Figure 3a). The best [Zn_3−Al−Cl]/CHT weight ratio was found to be 4:1. A slight excess of [Zn_3−Al−Cl] in the hybrid film can maintain a hydrophilic microenvironment favorable to the enzymatic process, while with too much [Zn_3−Al−Cl], the biocompatibility of the enzyme matrix decreased, inducing the poor analytical performance of the biosensor.

This hypothesis was further confirmed via the static contact angle measurements of the enzyme immobilization matrix (results shown in Figure 3b). The contact angle of pure CHT was measured to be 94.8 ± 1°. While the contact angle of the enzyme matrix decreased initially with increment of the [Zn_3−Al−Cl]/CHT weight ratios from 1 to 4, the minimum contact angle (37.4 ± 2°) can be observed at the ratio of 4. This implied that, at a ratio of 4, the hybrid matrix held highly hydrophilic characteristics.

In our previous work, the optimum ratio between [Zn_3−Al−Cl] and PPO is 1.[24] Thus, in this work, the film thickness can be adjusted easily by varying the total amount of the CHT/[Zn_3−Al−Cl]/PPO (1:4:4) mixture. An initial increase in response to 10 μM catechol was observed to be 16 μg of PPO loading at which point the response decreased gradually (Figure 4). An increasing thickness increases the amount of active PPO. 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. However, a thick CHT/[Zn_3−Al−Cl]/PPO film is not beneficial for an electrode response as the increase in the diffusion barrier. Therefore, this configuration with 16 μg of PPO was chosen for further experiments.

Optimization of Measurement Variables. The experimental variables, which can affect the amperometric determination of catechol, include the pH of supporting electrolyte, applied potential, and temperature of the system. The pH dependence of the CHT/[Zn_3−Al−Cl]/PPO electrode over the pH range 5.0−8.0 in PBS in the presence of 10 μM catechol was studied. As can be seen in Figure 5a, the electrochemical response is quite good at pH values ranging from 5.0 to 7.0 and the maximum current response was obtained at pH 6.0. This pH effect is similar to the wide optimal pH range of 5.0−8.0 reported for the free PPO.[29] However, PPO would lose activity irreversibly at the lower or higher pH values.[30,31] Therefore, PBS at pH 6 was selected as the electrolyte in subsequent experiments.

The dependence of the biosensor on the applied potential for amperometric signal of the CHT/[Zn_3−Al−Cl]/PPO sensor is displayed in Figure 5b. In the range between 0.1 and −0.3 V, the maximum response was obtained at −0.2 V, while a small decrease in biosensor response was observed for more negative potentials. This may be attributed to an increase in the direct reduction of oxygen at the electrode surface, leading to oxygen depletion within the biocoating and hence to a decrease in the enzymatic rate. Thus, −0.2 V was selected as the applied potential in subsequent experiments.

The effect of temperature on the biosensor response to 10 μM catechol is shown in Figure 6. The response first increases

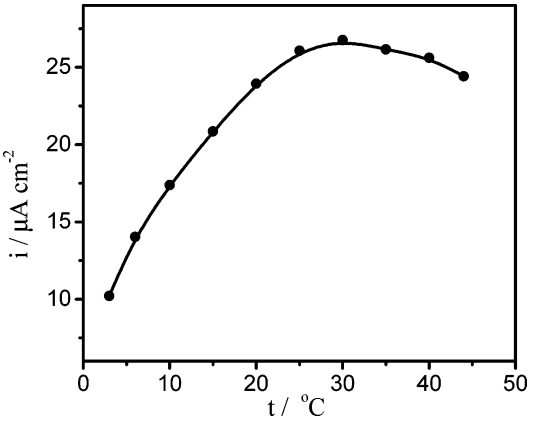

Figure 6. Influence of temperature on the biosensor response in 0.1 M PBS containing 10 μM catechol with pH 6.0; $E_{app} = -0.2$ V.

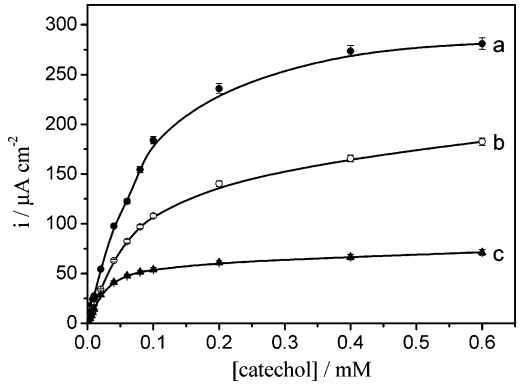

Figure 7. Calibration curves of CHT/[Zn_3−Al−Cl]/PPO (a), [Zn_3−Al−Cl]/PPO (b), and CHT/PPO (c) for catechol, in 0.1 M PBS (pH 6.0) at 25 °C, $E_{app} = -0.2$ V).

with increasing temperature from 3 to 30 °C and then decreases as the temperature increases further. Maximum response appears at about 30 °C. At higher temperatures, the current response decreases slowly due to the denaturation of the enzyme. For practical convenience, all other experiments were carried out at 25 °C.

The dependence of current on temperature range 3−25 °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, was 27.6 kJ mol^{-1} (the standard deviation is 1.5 kJ mol^{-1}), which is similar to that (24 ± 2 kJ mol^{-1}) of the [Zn_3−Al−Cl]/PPO electrode.[24]

Amperometric Biosensor Characteristics. Under optimal conditions in the determination of the presence of catechol (10 μM), the response time (defined as the time when 95% of the steady-state current is reached) was about 5 s. Such a rapid response indicates a fast mass transfer of the substrate across the film and a fast electron exchange between PPO and its substrates, indicating that the catalytic properties of the enzyme was not hindered by the hybrid CHT/[Zn_3−Al−Cl] matrix.

Figure 7 shows the typical calibration plots of CHT/[Zn_3−Al−Cl]/PPO (curve a), [Zn_3−Al−Cl]/PPO (curve b), and CHT/PPO (curve c) for the determination of catechol, respectively. Among the three PPO electrode, CHT/[Zn_3−Al−Cl]/PPO exhibits the best analytical performance. The linear range spans the concentration of catechol from 3.6 nM to 40 μM with a correlation coefficient of 0.998. Its sensitivity and I_{max} were 2750 ± 52 mA M^{-1} cm^{-2} and 408 μA cm^{-2}, respectively. This sensitivity for catechol is much higher than that of PPO immobilized in the polypyrrole−alginate conjugated system (350 mA M^{-1} cm^{-2}),[32] PPO entrapped electrochemically in the polyaniline film with the polyacrylonitrile-modified glassy carbon electrode (2030 mA M^{-1} cm^{-2}),[33] PPO incorporated with graphite-epoxy (1300 mA M^{-1} cm^{-2}),[34] and pyrrol−ammonium composite (1500 mA M^{-1} cm^{-2}).[31]Only a composite biosensor based on a carbon paste stabilized by an internally electropolymerized polypyrrole displays a higher catechol sensitivity, namely, 4700 mA M^{-1} cm^{-2}.[35] However, this result may be ascribed to a volume effect of the carbon paste and hence cannot be accurately compared to conventional biosensors exhibiting a defined thickness. The perfect sensitivity of the CHT/[Zn_3−Al−Cl]/PPO sensor may be attributed to the combined merits of chitosan and LDHs, such as high biocompatible and hydrophilic microenvironment, high permeability, and excellent adsorption ability. The apparent Michaelis−Menten constant (K_M^{app} = 0.13 mM) was evaluated from the electrochemical Lineweaver−Burk plot analysis of the catechol calibration curve. The K_M^{app} value is lower than that found for the free enzyme in solution (0.28 mM).[36] The low K_M^{app} values can be explained by the fact that *o*-quinone can enter into another enzymatic oxidation, providing a local increase in substrate concentration and an amplification of the electrode response. The immobilization of the PPO mentioned above appears to be beneficial to for improving the biosensor's performance.

The reproducibility of the analytical response obtained from different electrodes constructed by the same procedure was analyzed. Six different electrodes were tested independently for these amperometric responses to 10 μM catechol. The results revealed that the RSD value for all six electrodes was 4.8%. This indicates, in particular, an efficient and reproducible immobilization process of PPO within the hybrid CHT/[Zn_3−Al−Cl] matrix, even though the procedure used was a nonautomatic handmade process.

Both operational and long-term storage stability are very important from the practical application point of view, so these two parameters were also examined. The operational stability of the CHT/[Zn_3−Al−Cl]/PPO electrode was investigated by consecutive measurements of its response to 10 μM catechol. About 80% of the initial activity had been retained after 40 successive measurements. The long-term stability of the CHT/[Zn_3−Al−Cl]/PPO electrode stored at 4 °C was evaluated periodically using the same PBS containing 10 μM catechol. The biosensor exhibited excellent stability for 35 days; no loss of activity of the immobilized enzyme was observed. It still retained about 76% of its original response after 63 days of storage. This biosen-

Table 1. Response Characteristics of the CHT/[Zn_3−Al−Cl]/PPO Bioelectrode to Phenolic Compounds

phenolic compound	linear range (M)	correlation coefficient	sensitivity (mA M^{-1} cm^{-2})	detection limit (nM)	K_M^{app} (mM)	K_M^{app*} (mM)[a]
catechol	3.6×10^{-9} to 4×10^{-5}	0.998	2750 ± 52	0.36	0.13	0.22
p-cresol	4.0×10^{-9} to 2×10^{-5}	0.999	2490 ± 46	0.40	0.062	0.11
phenol	5.0×10^{-9} to 2×10^{-5}	0.999	2229 ± 12	0.50	0.053	0.068
m-cresol	7.0×10^{-9} to 2×10^{-5}	0.998	1537 ± 32	0.70	0.021	0.036

[a] K_M^{app*}: the values were obtained by [Zn_3−Al−Cl]/PPO electrode.

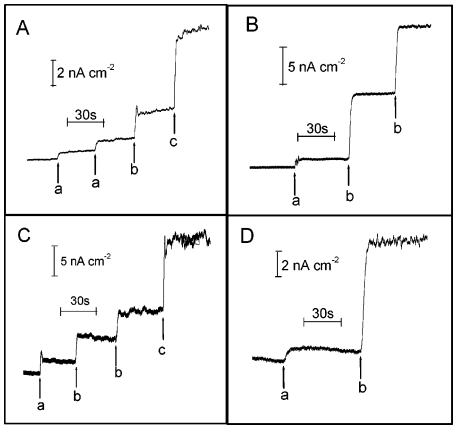

Figure 8. Actual current traces of CHT/[Zn_3–Al−Cl]/PPO bioelectrode for sub-nanomolar amperometric determination of phenolic compounds (at 25 °C, $E_{app} = -0.2$ V). (A) Successive addition of 1 μL (a), 2 μL (b), and 5 μL (c) of 3.6 μM catechol to 10 mL of PBS (pH 6.0). (B) Successive addition of 1 μL (a) and 10 μL (b) of 4.0 μM *p*-cresol to 10 mL of PBS (pH 6.0). (C) Successive addition of 1 μL (a), 5 μL (b), and 10 μL (c) of 5.0 μM phenol to 10 mL of PBS (pH 6.0). (D) Successive addition of 1 μL (a) and 10 μL (b) of 7.0 μM *m*-cresol to 10 mL of PBS (pH 6.0).

sor stability is much better than that of the [Zn_3−Al−Cl]/PPO electrode for which the residual activity is 60% after 28 days.[24] The superior stability can be attributed to the favorable microenvironment provided by the composite CHT/[Zn_3−Al−Cl] matrix. In addition, the adhesive ability and mechanical strength of the biomembrane can be effectively enhanced.

Response Characteristics of the Biosensor to Various Phenolic Compounds. Four phenolic compounds (catechol, *p*-cresol, *m*-cresol, and phenol) were determined by the CHT/[Zn_3−Al−Cl]/PPO electrode. The response characteristics of the biosensor, including linear range, correlation coefficient, sensitivity, detection limit, and the K_M^{app} value for the different phenolic compounds, are summarized in Table 1. The actual current traces of CHT/[Zn_3−Al−Cl]/PPO bioelectrode for sub-nanomolar amperometric determination of phenolic compounds are shown in Figure 8. The biosensor shows sensitivity for the substrates in the following order: catechol > *p*-cresol > phenol > *m*-cresol. This sequence is the same as that reported for LDH-modified PPO electrode.[24] The K_M^{app} value gives information on the enzyme−substrate kinetics for the enzyme electrode. The K_M^{app} values obtained by CHT/[Zn_3−Al−Cl]/PPO electrode are 0.13, 0.062, 0.053, and 0.021 mM for catechol, *p*-cresol, phenol, and *m*-cresol, respectively. These values are smaller than those recorded with the [Zn_3−Al−Cl]/PPO electrode (shown in Table 1), respectively. Moreover, these values are lower than those reported for the free enzyme. This reflects either a preconcentration effect of the composite matrix on the enzyme substrate or an electroenzymatic recycling phenomenon.[37]

Conclusion

An enzyme electrode based on the immobilization of PPO in a new CHT/LDHs bioinorganic composite film was developed. The biosensor exhibited a variety of good analytical performances including high sensitivity, good repeatability and reproducibility, rapid response, and storage stability. These advantages can be attributed to the coimmobilization of CHT within the LDHs gel, which highly improves the adhesive ability, biocompatibility and the mechanical strength of the host matrix for enzyme. In addition, the usage of glutaraldehyde, which can make enzyme denatured, could be avoided. Therefore, it can be extended to immobilize other enzymes and biomolecules, which will greatly facilitate the development of biosensors and other bioelectrochemical devices.

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