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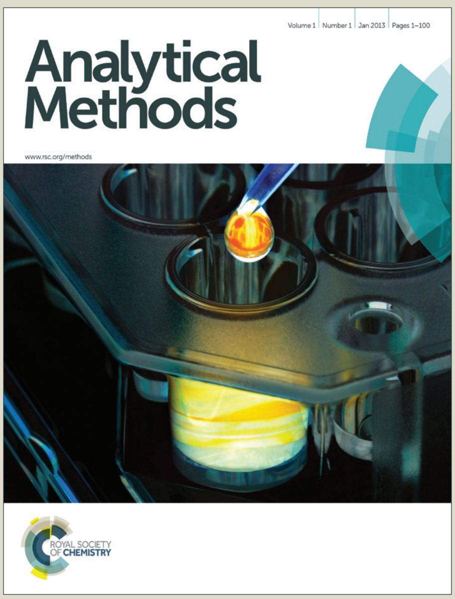

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ARTICLE TYPE

Direct electron transfer of hemoglobin intercalated in exfoliated Ni-Al-CO_3 layered double hydroxide and its electrocatalysis to hydrogen peroxide

Yu-Ge Liu, * Xiu-mei Zhang, Xing-hao Tu, Chang-bin Wei, Ling-ling Lv

Key Laboratory of Tropical Fruit Biology of Ministry of Agriculture, The South Subtropical Crop Research Institute, Chinese Academy of Tropical Agricultural Science,Zhanjiang, 524091, China

In this study, hemoglobin (Hb) was entrapped into the exfoliated Ni-Al-CO_3 layered double hydroxides (LDH). UV-vis spectra analysis displayed that no significant denaturation occurred to the protein. Electrochemical results showed that exfoliation of LDH enhanced the direct electron transfer between protein molecules and electrode, and the entrapped protein showed high bioactivity in a wide range of pH values. A pair of well-defined redox peaks was observed at -0.39 and -0.33 V on the glassy carbon electrode (GCE) modified with the Hb/LDH composite. The electrode reactions showed a surface-controlled process with a single electron transfer at the scan rate from 100 to 400 mV/s. The sensor constructed displayed excellent response to the reduction of hydrogen peroxide (H_2O_2) with wide linear range, low detection limit and good stability. The modified electrode can also be used for the reduction of oxygen.

Introduction

The determination of hydrogen peroxide (H_2O_2) is of great importance in many fields, such as food, pharmaceutics, industry, clinical laboratory and so on. [1-2] Numerous quantitative methods have been developed for the detection of H_2O_2. The most commonly used approaches include spectrometry [3, 4], chemoluminescence [5-6] and amperometry. [7-8] However, these methods are either time-consuming or require expensive reagents and equipments. In recent years, much attention has been paid to the amperometric detection of H_2O_2 due to its simplicity, high selectivity and high sensitivity. [9-11] This was generally based on the sensors constructed from the direct electrochemistry of proteins and enzymes.

Biosensors based on the direct electron transfer between redox proteins and electrode surface have aroused great interest since the realization of reversible electrochemistry of cytochrome c on modified electrodes in 1977. [12] Besides, such sensors can also be used in the fields of clinical diagnosis, [13] food analysis, [14] and so on. For the application of biosensors, proteins should be immobilized on the electrode surface to avoid interference.

However, the electroactive centers of redox proteins are often embedded within the structure of biomacromolecules, and the direct electron transfer between the electroactive center and the substrate electrode is difficult to occur. Meanwhile, adsorption of protein molecules onto bare electrode surface may lead to their denaturation, which also decreases direct electron transfer rate and the efficiency for detecting H_2O_2. Therefore, immobilization of proteins on supports is needed to display their special properties.

Different kinds of materials, including nanomaterials, biopolymers, ionic liquids, hydrogel, and so on, have been used for the decoration of electrodes to provide specific microenvironments and properties. This was favorable for the maintenance of bioactivity and realization of direct electrochemistry of proteins.

Recently, layered materials have attracted great attention for their application in the immobilization of proteins and detection of H_2O_2. The "flexible pores" and the interlayer galleries in layered materials can be used to hold the dimension of guests, which makes them quite suitable to immobilize proteins with different dimensions. [15] Different kinds of layered materials have been reported as supporting matrices for proteins, such as layered niobate $HCa_2Nb_3O_{10}$, [16-17] layered polysilicate magadiite, [18] layered titanate [19] and layered phosphates.[20]

Layered double hydroxides (LDHs) belong to one kind of the most useful inorganic layered compounds. The LDHs can be described as $\left[M^{II}_{1-x}M^{III}_{x}(OH)_2\right]^{x+}\left(A^{n-}_{x/n}\right)^{x-}\cdot mH_2O$, where M^{II} is a divalent cation such as Mg^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, etc., M^{III} is a trivalent cation such as Al^{3+}, Ga^{3+}, Fe^{3+}, Co^{3+}, Mn^{3+}, Cr^{3+}, etc., and A^{n-} is an n-valent anion such as Cl^-, NO^{3-}, SO_4^{2-}, CO_3^{2-}, etc.

LDHs have net positive charges of the layer balanced by exchangeable anions intercalated between the sheets. This kind of compounds can be used to fabricate biosensors with excellent performances due to the special properties they possess, including wide interlayer composition, high and tunable layer charge density, and opened layer structure. The direct electron transfer of heme proteins have been realized on Zn-Al-SDS and NiAl-NO_3 LDHs by Li et al.[21-22] The direct electrochemistry of horseradish peroxidase (HRP) and hemoglobin (Hb) was achieved directly at Ni-Al-NO_3 and Mg-Al-Cl LDH, [23-24] and the adsorption of myoglobin (Mb) was studied on Ni-Al-Br LDH colloid suspension.[25]

However, the application of LDHs in biosensors may be

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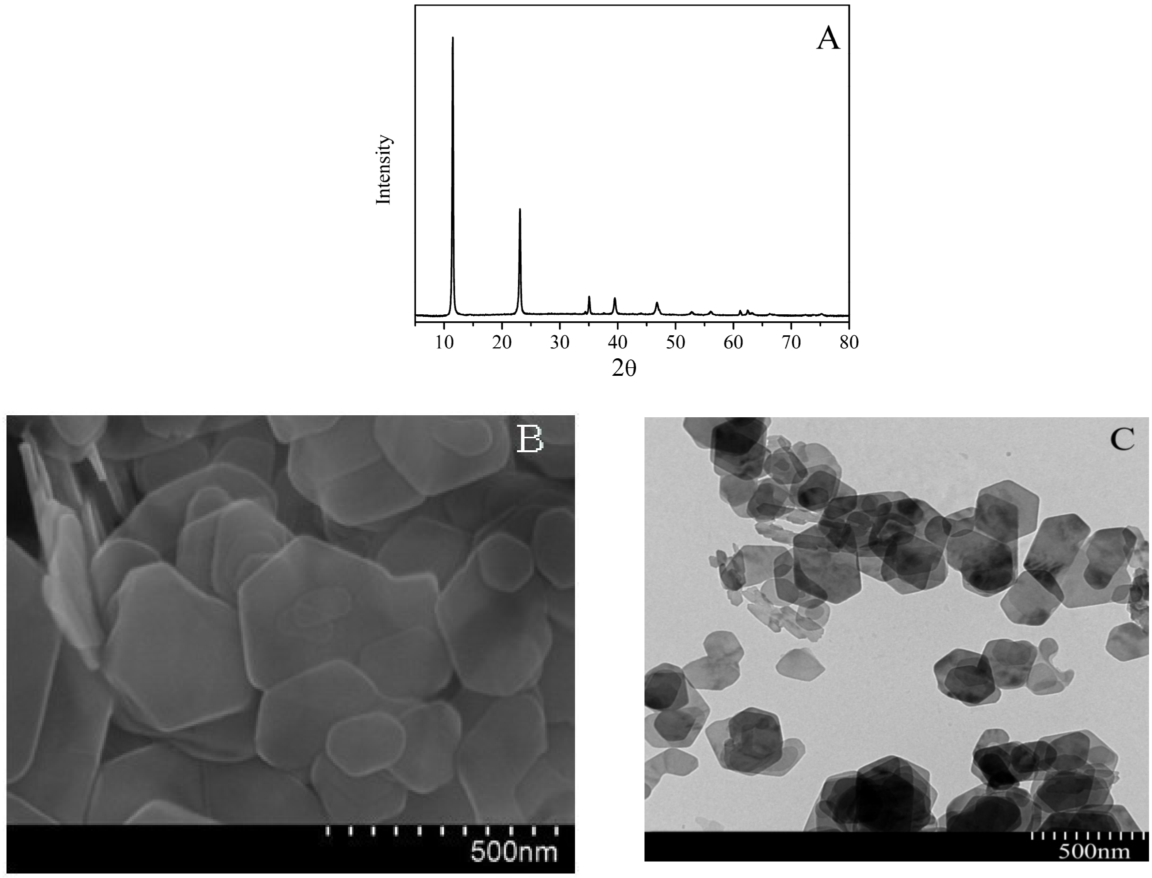


Fig. 1 (A) XRD pattern, (B) SEM image and (C) TEM image of prepared Ni-Al-CO_3 LDH

limited due to the narrow distance between the layers and the small surfaces areas. Exfoliated layered materials not only have larger surface areas but also possess some other specific properties which are beneficial for their application in biosensors. Several layered materials have been in this field, such as α-zirconium phosphate, [20, 26] HNb_3O_8 [27] and $HCa_2Nb_3O_{10}$. [17]

In the present work, pre-exfoliated Ni-Al-CO_3 LDH was used for the immobilization of Hb and the construction of a sensor to detect H_2O_2. Compared with other layered materials, Ni-Al-CO_3 LDH possesses some unique electrochemical performances and could provide a more suitable microenvironment to immobilize proteins used for biosensors. This may be due to the inclusion of two different metals and the substitution of a fraction of the Ni(II) by Al(III). The direct electron transfer between the electrode and the protein was realized. The immobilized protein possessed a much higher direct electron transfer constant compared with that use unexfoliated LDH. The sensor fabricated displayed fast amperometric response, low detection limit and good stability for the detection of H_2O_2.

Experimental section

Reagents

Bovine heart hemoglobin was purchased from Sigma and used without further purification. Formamide and hydrogen peroxide (H_2O_2, 30 wt% solution) were purchased from Sinopharm Chemical Reagent Co., Ltd. All the other reagents are of analytical grade and used as received. Ultra-pure water was used for the preparation of solutions.

Synthesis and exfoliation of Ni-Al-CO_3 LDH

The synthesis of Ni-Al-CO_3 LDH was performed according to the procedure described before.[28] Generally, a mixture of $NiCl_2$ (0.1 M), $AlCl_3$ (0.05 M), and urea (0.15 M) was hydrothermally treated at 190 °C for 2 days. The resulted material was collected by centrifuging.

The material was first treated by a HCl-NaCl solution into their NO_3^- form before exfoliation. Then, 0.1 g treated LDH was mixed with 100 ml of formamide in a sealed beaker, which was purged with N_2 in advance. The mixture was stirred vigorously at room temperature for 48 h. The resulted colloidal suspension was

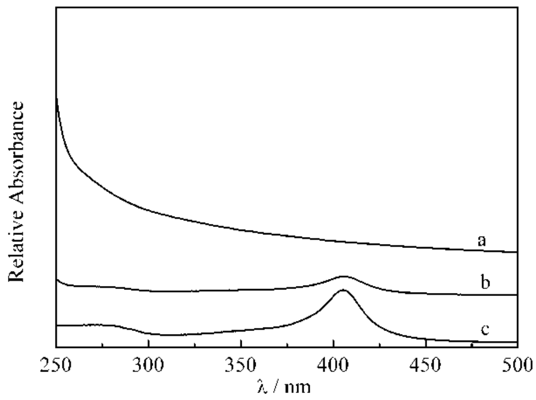


Fig. 2 UV-vis spectra of (a) pre-exfoliated LDH, (b) Hb/LDH and (c) Hb in 0.1 M PBS7.0.

used without further treatment.

Entrapment of Hb and preparation of composite modified electrode

For the entrapment of Hb, stock solution of the protein (2 mg/mL, 0.1 M phosphate buffer solution (PBS), pH 7.0) and the exfoliated LDH were mixed together in a 1:1 volume ratio. The mixture was equilibrated for 24 h at room temperature and the resulted suspension was directly used for further test.

Glass carbon electrode (GCE) was polished with 1.0, 0.3 and 0.05 μm alumina powder successively, followed by rinsing thoroughly with ultra-pure water. The polished electrode was then sonicated in acetone and ultra-pure water and finally allowed to dry at room temperature. 10 μL of the suspension of Hb/LDH achieved above was deposited onto the surface GCE. The electrode was then left to dry at 4 ^{o}C for at least 24 h. The sensor was stored under the same condition when not used.

Apparatus and measurements

X-ray diffraction (XRD) data was recorded on a PANalytilal X'pertpowder diffractometer equipped with Cu Kα radiation (λ= 0.154 nm). Transmission electronmicroscopy (TEM) was investigated on Hitachi HT7700. Scanning electron microscope (SEM) images were taken on Hitachi S-4800 field-emission microscope. UV-vis absorption spectra were obtained on a Shimadzu UV-2700 spectrophotometer. Cyclic voltammetric and amperometric experiments were conducted with a CHI660B workstation (Shanghai Chenhua, Shanghai). All experiments were carried out using a conventional three-electrode system, where GCE modified with Hb/LDH as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode as reference electrode. All solutions were deoxygenated by highly pure nitrogen before and during the measurements.

Results and discussion

SEM analysis

The XRD pattern of the synthesized Ni-Al LDH (Fig. 1A) showed very intense basal reflection series, indicating highly crystalline nature of the material. The 003 reflection was located at a 2θ angle of about 11.48°, indicating a basal spacing of 0.77

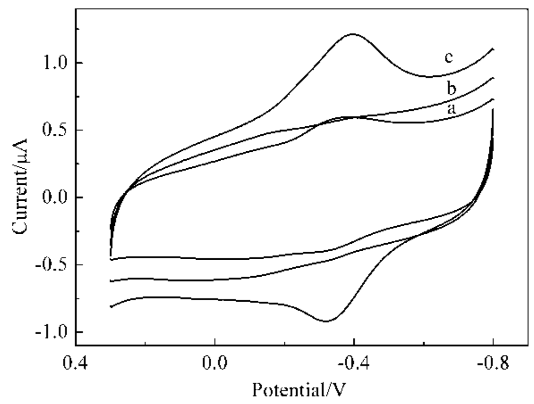


Fig. 3 Cyclic voltammograms of (a) Hb, (b) exfoliated LDH and (c) Hb/LDH composite modified electrodes at 100 mV/s in 0.1 M PBS 7.0

nm. It could be seen from SEM and TEM images that the material was quasi-hexagonal platelets with widths between 200 and 400 nm and thickness of about 30 nm (Fig. 1B, C). The shape was quite similar to that reported. [28]

UV-vis absorption spectroscopic analysis

UV-vis spectroscopic analysis is quite useful for monitoring the possible change of Soret absorption band in the heme group region. [29] The band shift may provide some information for the possible denaturation of heme protein, particularly that of conformational change. Shown in Fig. 2 are the UV-vis spectra of exfoliated LDH, Hb/LDH and Hb solutions in 0.1 M PBS7.0, respectively. It can be clearly seen that free Hb (curve c) and Hb/LDH (curve b) have Soret absorptions at 405 and 406 nm, while there was no adsorption in the field studied for the exfoliated LDH UV-vis spectrum (curve a). All this showed that the protein retained its bioactivity after composited with LDH.

Direct electron transfer of Hb/LDH modified electrode

Given in Fig. 3 are the cyclic voltammograms (CVs) of different electrodes at 100 mV/s in PBS 7.0. No peaks appeared at the electrode modified by exfoliated LDH (curve b), indicating that LDH was inelectroactive in the area discussed. When the electrode was modified with only Hb (curve a), only a reduction peak was observed, and the current decreased greatly with cycle numbers, suggesting that direct electron transfer was impossible between Hb and electrode without supports. However, a pair of well-defined redox peaks was observed at the Hb/LDH modified electrode at -0.39 and -0.33 V. These peaks were located much close to the characteristic potential of the heme Fe_{III}/Fe_{II} couples reported. [30] These results presented strong evidence that the direct electron transfer between Hb and GCE was achieved after combination with LDH, and the immobilization may have more favorable orientation and facilitate the direct electron transfer between protein and the electrode.

The CVs of Hb/LDH modified electrode displays a well-defined peak shape at different scan rates from 100 to 400 mV/s (Fig. 4). With the increase of scan rate, the redox peak currents of the Hb increased linearly (inset of Fig. 4), and the peak-to-peak

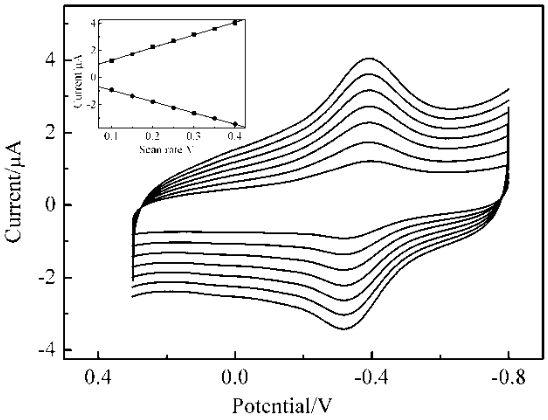


Fig. 4 Cyclic voltammograms of Hb/LDH composite modified electrode in 0.1 M PBS 7.0 at 100, 150, 200, 250, 300, 350 and 400 mV/s (from inner to outside). Inset: plot of peak current vs. scan rate.separation also increased, suggesting a surface-controlled process.

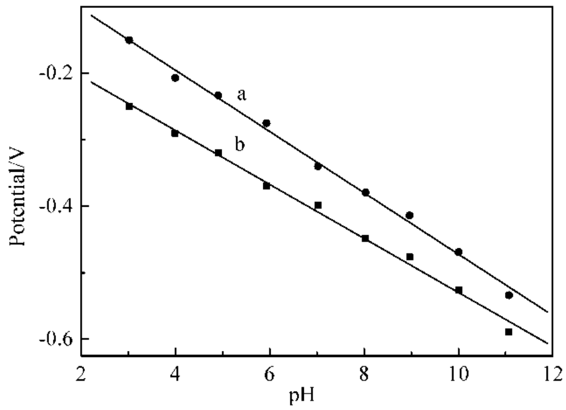


Fig. 5 Plots of pH vs. (a) cathodic, and (b) anodic potential.

For thin-layer electrochemistry, integration of CV peak can give the total amount of charge (*Q*) passed through the electrode for reduction or oxidation of electroactive species in the thin film. Its surface concentration (Γ^*) can be calculated from the Faraday's law:

$$\Gamma^* = Q/nFA$$

Where *n* is the number of electrons transferred, *F* is Faraday's constant, and *A* is the electrode area. The average surface coverage of Hb calculated from the Faraday's law is 5×10^{-11} mol/cm^2 for the Hb/LDH modified GCE. The value is larger than the theoretical monolayer coverage of Hb (ca. 1.89 $\times10^{-11}$ mol/cm^2) on the basis of its crystallographic dimensional structure, assuming more than one layer of Hb immobilized on the electrode took part in the electrode reaction. The bigger average surface coverage may be ascribed to the expanded interspace and surface area to hold more Hb molecules.

Small peak-to-peak separation always indicates a fast electron transfer rate. The electron transfer rate constant k_s can be estimated by the Laviron equation: [31]

$$\log K_s = \alpha\log(1-\alpha) + \alpha(1-\alpha)\log\alpha - \log RT/nFv - \alpha(1-\alpha)nF\Delta E_p/2.3RT$$

Where α is the charge-transfer coefficient, *R* is the gas constant, *T* is the absolute temperature, ΔE_p is the peak potential separation, and *v* is the scan rate. A graph of the peak potential versus the logarithm of the scan rate yields a straight line, from the slope a charge-transfer coefficient of 0.9 was estimated for Hb. The peak-to-peak separations were 65、69、73、89、92 mV at the scan rate of 100、150、200、250、300 mV/s, giving an average k_s value of 2.194±0.299 s. The value is much larger than those reported previously. [32-35] The electron transfer rate of Hb in exfoliated LDH was greatly increased compared with that of unexfoliated, [32] suggesting that exfoliation of LDH could provide a specific microenvironment that facilitated the electron transfer between Hb and the electrode.

Influence of solution pH

In most cases, the pH values of solutions are quite essential to the electrochemical behaviors of proteins. In this research, the Hb/LDH modified electrode showed strong dependence on solution pH. All the changes in the peak potential and current caused by pH (from 3 to 11) were reversible. For example, the cyclic voltammogram for the Hb/LDH at pH 7 was reproduced after immersion in pH 4 buffer and then returned to the pH 7 buffer. The cathodic, anodic potential for the Hb/LDH electrode showed a linear relationship with pH in a wide range from 3 to 11 with slopes of -40.63 and -46.13 mV pH^{-1} (Fig. 5), suggesting that there was nearly one electron participated in the electron transfer process. Thus, the reaction equation for the electrochemical reduction of Hb may be described as follows: [36]

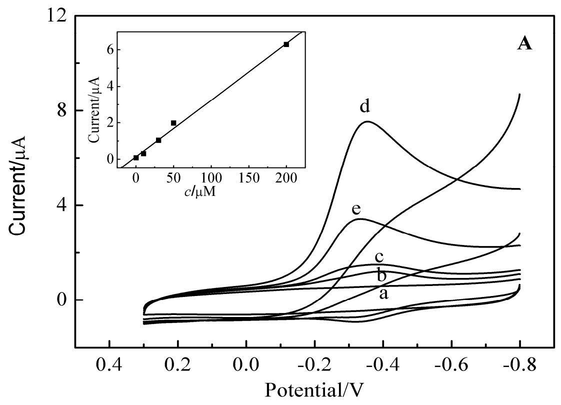
$$\text{Hb heme Fe(III)} + H^+ + e^- \rightarrow \text{Hb heme Fe(II)}$$

Electrocatalytic behavior of the immobilized Hb

It is well known that proteins and enzymes containing the heme group have the ability to reduce hydrogen peroxide (H_2O_2) electrocatalytically, which means a biosensor for detecting H_2O_2 can be fabricated on the basis of the excellent performance of Hb/LDH composite.

Shown in Fig. 6A are the CVs of Hb/LDH and LDH modified electrodes in 0.1 M pH 7.0 PBS before and after the addition of H_2O_2. No current is observed on LDH modified electrode. However, it can be clearly seen that the reduction peak current increased and the anodic peak current decreased dramatically with the addition of H_2O_2 on the Hb/LDH modified electrode. Besides, the currents of the reduction peaks increased with the increase of concentration, indicating a typical electrocatalytic reduction process. The reduction peak currents were in line with the concentration of H_2O_2 within the range of 0.4-200 μM with a detection limit of 0.15 μM (*N* = 5; *R* = 0.998; inset of Fig. 6A). The relative standard deviation (RSD) of the peak current in six successive determinations at a H_2O_2 concentration of 50 μM was 3.22% for Hb/ LDH modified GCE.

Additionally, the modified electrode can also be used for the reduction of O_2. When air was injected into PBS, the reduction current increased (Fig. 6B). This increase in the reduction peak was accompanied by the decrease of the oxidation peak because Fe(II) in Hb had reacted with oxygen. An increase in the amount of air in solution resulted in the increase of the reduction peak



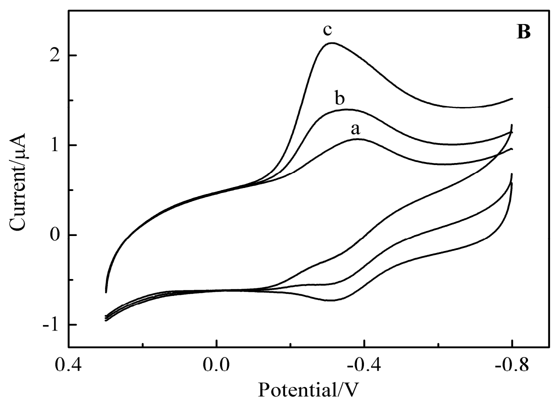


Fig. 6 (A) Cyclic voltammograms of Hb/LDH composite modified electrode without (a) and with (b) 1×10^{-5} M, (c) 5×10^{-5} M, and (d) 2×10^{-4} M of H_2O_2 at 100 mV/s in 0.1 M PBS 7.0. Inset: Calibration curve of current vs. H_2O_2 concentration. (B) Cyclic voltammograms of Hb/LDH composite modified electrode without (a) and with (b) 5 mL and (c)10 mL of air at 100 mV/s in 0.1 M PBS 7.0.

current.

The apparent Michaelis-Menten constant k_m^{app}, which gives an indication of the enzyme-substrate kinetics, is generally used to estimate the biological activity of immobilized enzyme. This constant was calculated by Lineweaver-Burk equation:[37]

$$1/I_{ss}=1/I_{max}+k_m^{app}/I_{max}C$$

Where I_{ss} is the steady current after the addition of substrate (which can be obtained from amperometric experiments), C is the bulk concentration of the substrate, and I_{max} is the maximum current measured under the saturated substrate condition. The apparent Michaelis-Menten constant was calculated to be 267 μM for the Hb/LDH composite modified electrode. The value was smaller than that of Hb–HCa_2Nb_3O10 and Hb–PHB film modified electrode, [17, 38] suggesting a higher affinity and enzymatic activity.

Stability and reproducibility of the Hb/LDH modified electrode

Additional experiments were carried out to test the reproducibility and stability. No obvious change was found after the Hb/LDH modified electrode was immersed in PBS and stored in the refrigerator at 4 °C for 20 h. The peak current retained 99% of its initial response for Hb/LDH modified GCE after 100 cycles at a H_2O_2 concentration of 20 μM at 100 mV/s. The biosensor could keep 88% of its initial response to 20 μM of H_2O_2 in a dry state at 4 °C within two weeks. Five different biosensors were made one by one in five separate days. The relative standard deviation (RSD) of the peak currents to 50 μM H_2O_2 for the five biosensors was 2.5%. All this indicated a good stability of the biosensor.

Conclusion

Ni-Al-CO_3 LDH was prepared and exfoliated for the immobilization of Hb on glass carbon electrode. Results showed that the protein retained its bioactivity and the direct electron transfer between Hb and the electrode was realized. The exfoliation of LDH increased the direct electron transfer greatly compared with that of unexfoliated, and the entrapped protein remained its bioactivity within a wide pH range. The sensor constructed showed fast detection to the reduction of H_2O_2 with a wide linear range and low detection limit, and it also can be used for the reduction of oxygen. This work reveals that exfoliated LDH provides a promising platform for the immobilization of proteins on electrodes and development of biosensors with excellent performances.

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Entry for the Table of Contents

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