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Horseradish peroxidase immobilization on carbon nanodots/CoFe layered double hydroxides: Direct electrochemistry and hydrogen peroxide sensing

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ABSTRACT

Carbon nanodots and CoFe layered double hydroxide composites (C-Dots/LDHs) were prepared via simply mixing C-Dots and CoFe-LDHs. The as-prepared composites were used for the immobilization of horseradish peroxidase (HRP) on the glass carbon (GC) electrode. The electrochemical behavior of the HRP/C-Dots/LDHs/GC electrode and its application as a H_2O_2 biosensor were investigated. The results indicated that HRP immobilized by C-Dots/LDHs retained the activity of enzyme and displayed quasi-reversible redox behavior and fast electron transfer with an electron transfer rate constant k_s of 8.46 s^{-1}. Under optimum experimental conditions, the HRP/C-Dots/LDHs/GC electrode displayed good electrocatalytic reduction activity and excellent analytic performance toward H_2O_2. The H_2O_2 biosensor showed a linear range of 0.1–23.1 μM (R^2=0.9942) with a calculated detection limit of 0.04 μM (S/N=3). In addition, the biosensor exhibited high sensitivity, good selectivity, acceptable reproducibility and stability. The superior properties of this biosensor are attributed to the synergistic effect of HRP, C-Dots and CoFe-LDHs, which has been proved by investigating their electrochemical response to H_2O_2. Thus the C-Dots and LDHs composites provide a promising platform for the immobilization of redox enzymes and construction of sensitive biosensors.

1. Introduction

Electrochemical biosensors based on the direct electron transfer of redox enzymes have attracted great attention due to their excellent selectivity, sensitivity and conveniences (Gholivand and Khodadadian, 2014). They find wide applications in many fields such as pharmaceutical monitoring, clinical diagnosis and food analysis (Song et al., 2014).

However, there are two key problems in fabricating such kinds of biosensors. The first problem is how to immobilize enzymes onto the electrode without denaturation. And the second one is how to accelerate the electron transfer between the redox proteins and the electrode. Since the redox centers of most enzymes are buried deeply in their 3D matrix structure, their direct electrochemistry is often difficult to be realized (Wan et al., 2013). The solution for the two key problems is to select appropriate material for the design of electrochemical biosensors.

Many nano-materials such as metal nanoparticles (Chen et al., 2012), metal sulfide (Fan et al., 2014), metal oxides (Yang et al., 2014; Yagati et al., 2013) and carbon materials (Fang and Wang, 2013) have been used for immobilizing the biomolecules due to their high surface area and good biocompatibility. However, sometimes one nano-material alone does not realize the two functions discussed above simultaneously. Therefore, hybrid materials with ensemble effects may be promising candidates for direct electrochemistry of redox enzymes. Recently the electrochemical biosensors based on hybrid material platform have received considerable attention (Mao et al., 2013; Shan et al., 2009; Sheng et al., 2013; Lu et al., 2013). Compared with the corresponding single material, the hybrid materials such as Ag@C core–shell (Mao et al., 2013), graphene/ionic liquid (Shan et al., 2009), fullerene–nitrogen doped carbon nanotubes (Sheng et al., 2013), and cyclodextrin/graphene (Lu et al., 2013) facilitated the electron transfer of enzymes more effectively and brought better analytic performances of the biosensors.

Carbon nanodots (C-Dots), namely fluorescent carbon nanoparticles, have received considerable attention since they were reported in 2004 (Xu et al., 2004). Now the investigations on C-Dots are focused on their applications in bio-imaging due to their excellent biocompatibility, high quantum yield, and good

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photostability (Cao et al., 2007; Baker and Baker, 2010; Wang et al., 2013). However, their applications in electrochemistry are barely explored. It has been reported that many carbon materials (Ambrosi et al., 2014; Wang et al., 2011) can enhance the electron transfer of redox enzymes (Mao et al., 2013). Compared with them, C-Dots are small (below 10 nm) and functionalized with rich carboxylic/carbonyl group which may be more beneficial to the interaction of C-Dots and HRP and thus promotes the direct electron transfer of HRP better (Shi et al., 2011). Moreover, the preparation of C-Dots is simple and of low-cost which meets requirements of practical electrochemical biosensors. Therefore it is an interesting work to explore the application of C-dots in electrochemical biosensors. However, in order to retain the physical stability of the biosensor, C-dots should be immobilized on the electrode, avoiding their loss due to the small size and their hydrophilic surface.

Layered double hydroxides (LDHs) are a class of layered anionic clays which are composed of positively-charged brucite-like layers and interlayer anions along with water molecules. LDHs have been widely used for the immobilization of enzymes due to their excellent adsorption ability and biocompatibility (Mousty and Prévot, 2013; Mousty, 2010). Especially, it has been proved by our group that Fe-based LDHs are more suitable for the direct electron transfer of heme proteins compared with Al-based LDHs (Li et al., 2012). Thus CoFe-LDHs were selected for the co-immobilization of C-dots and enzyme to fabricate the biosensor.

In the present work, an electrochemical H_2O_2 biosensor was designed by immobilizing horseradish peroxidase (HRP) using C-Dots and CoFe-LDHs composites (denoted as C-Dots/LDHs) on the surface of GC electrode. The direct electrochemistry behavior of HRP in the C-Dots/LDHs was investigated in detail. The prepared biosensor exhibited good electrocatalysis and analysis properties toward H_2O_2.

2. Experimental

2.1. Reagents and chemicals

Horseradish peroxidase (HRP) was obtained from Sangon Biotech Co., Ltd. (China). All other reagents were of analytical purity and used without further purification. Milli-Q purified water (Millipore, $\geq 18.2\ M\Omega$ cm) was used throughout the study.

2.2. Instrumentation

Powder X-ray diffraction (XRD) data were recorded by a Shimadzu XRD 6000 X-ray diffractometer (Shimadzu, Japan) based on Cu Kα radiation ($\lambda = 0.15406$ nm). The morphologies of LDH were examined with a Hitachi S-4800 scanning electron microscope (SEM, Hitachi, Japan). The emission spectra of carbon dot were obtained using a Hitachi F-4500 luminescence spectrometer (Hitachi, Japan). UV–visible (UV–vis) absorption spectroscopy measurement was performed on a UV-2450 spectrophotometer (Shimadzu, Japan).

All electrochemical experiments were carried out on a CHI660C electrochemical workstation (CH Instruments, Shanghai, China). A conventional three-electrode system was employed with a platinum wire as the counter-electrode, a saturated calomel electrode (SCE) as the reference electrode, and a modified (or bare) glassy carbon electrode (GCE) as the working electrode. All electrochemical experiments were performed at room temperature.

2.3. Preparation of CoFe-LDHs

CoFe-LDHs were synthesized by a modified coprecipitation method. Typically, a mixed salt solution containing Co^{2+} and Fe^{3+} was prepared by dissolving $CoCl_2 \cdot 6H_2O$ and $FeCl_3 \cdot 6H_2O$ in deionized water. The total concentration of Co^{2+} and Fe^{3+} was kept at 1 M with Co^{2+}/Fe^{3+} ratio of 2:1. Then a solution of NaOH (2 mol/L) was added dropwise to the mixed salt solution under vigorous stirring until the pH reached 9.5. After that the slurry reacted in an autoclave at 100 °C for 4 h. The resulting CoFe-LDHs were centrifuged, subsequently washed with water and anhydrous ethanol several times, and finally dried at 60 °C overnight in a vacuum oven.

2.4. Synthesis of C-Dots

Carbon nanodots (C-Dots) were prepared according to the previous report (Guo et al., 2013). Briefly, a mixed solution of sodium citrate (1.7 wt%) and NH_4HCO_3 (12.8 wt%) underwent a hydrothermal treatment at 180 °C for 4 h. Then the product was purified through a dialysis tube for about 24 h in dark.

2.5. Preparation of HRP/C-Dots/LDHs modified electrode

Prior to use, the bare GCE was carefully polished with alumina powder (1.0, 0.3, and 0.05 μm in order) on a wet polishing cloth and then thoroughly cleaned ultrasonically in acetone and water.

The HRP/C-Dots/LDHs modified electrode was prepared as follows. Firstly 1 mg mL^{-1} CoFe-LDHs suspension was prepared by dispersing CoFe-LDHs in deionized water with the aid of ultrasonication. Then 1 mL as-prepared CoFe-LDHs suspension was mixed with 100 μL C-Dots and the mixture was stirred for 24 h. After that, 100 μL 1 mg mL^{-1} HRP was added into the mixture suspension of C-Dots/LDHs with stirring for another 24 h. For the stability of modified electrode, 0.1 mL 5 wt% Nafion was mixed with the suspension of HRP/C-Dots/LDHs. Finally, 8 μL of this suspension was cast on the pretreated GCE surface and dried at room temperature for 6 h. In this way, a HRP/C-Dots/LDHs/GCE was obtained. For comparison, LDHs/GCE, HRP/LDHs/GCE, C-Dots/LDHs/GCE, and C-Dots/GCE were prepared in a similar way.

3. Results and discussion

3.1. Characterization of HRP/C-Dots/LDHs

CoFe-LDHs were prepared by the coprecipitation method. Fig. 1A shows the XRD pattern of CoFe-LDHs, which exhibits the characteristic reflection of LDH structure with a serial of (00*l*) peaks appearing. As shown, in addition to the peaks corresponding to the 003 (10.83°), 006 (23.41°), and 009 (34.04°) reflections of CoFe–Cl LDHs, no peaks of impurities were discerned, indicating the high purity of the products that were synthesized in the above mentioned system, which is in contrast to the impure products reported in the handbook (Braterman et al., 2004). The 003 reflections are all located on almost the 2θ angle of $\sim 10.9°$, indicating a basal interlayer spacing of ~ 0.78 nm for Cl-LDH, quite similar to those reported elsewhere (Liu et al., 2006; Ma et al., 2011). The SEM image in Fig. 1B displays that CoFe-LDHs consist of hexagonal platelets which aggregate into a 3D architecture with the width of about 500 nm and the thickness of about 60 nm. The open 3D architecture is thought to be helpful for the further immobilizing of C-Dots and HRP.

C-Dots were synthesized by the hydrothermal method from sodium citrate according to the literature (Guo et al., 2013). The

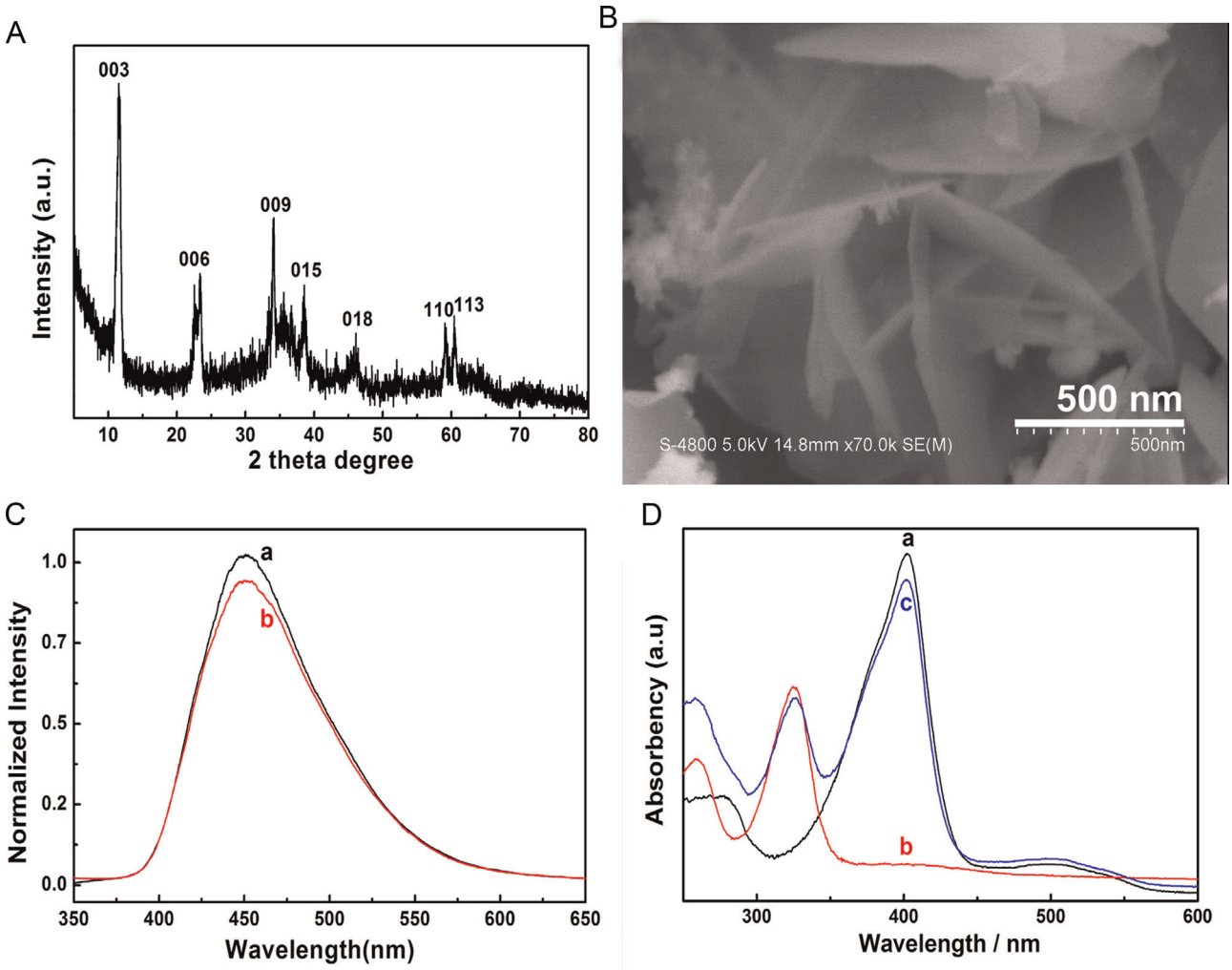

Fig. 1. (A) XRD pattern and (B) SEM image of CoFe-LDHs. (C) Emission spectra of (a) C-Dots and (b) C-Dots/LDHs. (D) UV–vis spectra of (a) HRP, (b) C-Dots, and (c) HRP/C-Dots/LDHs.

emission spectra of C-Dots are shown in Fig. 1C (curve a). When excited at 350 nm, the emission peak appears at 450 nm which is similar to the report. In addition, the composite of C-Dots/LDHs showed almost the same emission spectra as those of C-Dots (curve b in Fig. 1C), which indicated that the functional groups of C-Dots still remained active upon mixing with CoFe-LDHs.

The HRP/C-Dots/LDHs composites are characterized by UV–vis spectrometry and the results are presented in Fig. 1D. The composites exhibited two obvious absorption peaks at 325 nm and 403 nm (curve c). The absorption peak at 325 nm belongs to C-Dots (curve b) and the absorption peak at 403 nm is attributed to the characteristic Soret band of HRP (Mao et al., 2013; Guo et al., 2013). The same absorption wavelength of native HRP (curve a) and HRP in HRP/C-Dots/LDHs composites implies that the C-Dots/LDHs do not induce significant denaturation of HRP in phosphate buffer solution (PBS) and are suitable for the immobilization of HRP.

3.2. Electrochemical behavior of HRP/C-Dots/LDHs modified GC electrode

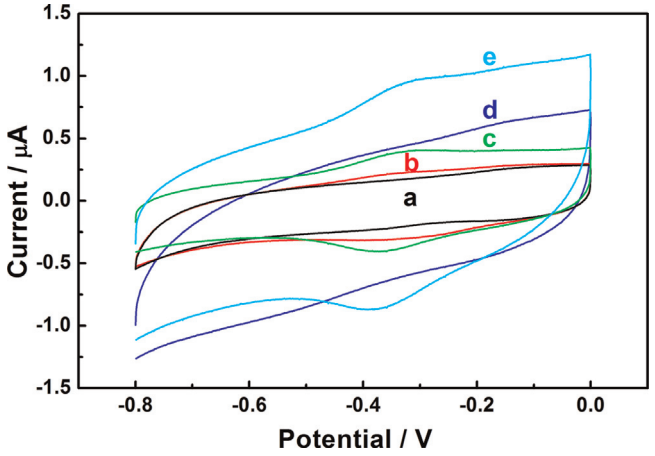

Fig. 2. Cyclic voltammograms of the (a) GC, (b) LDHs/GC, (c) HRP/LDHs/GC, (d) C-Dots/LDHs/GC, and (e) HRP/C-Dots/LDHs/GC electrodes in 0.1 M PBS (pH=7.0) at a scan rate of 50 mV s^{-1}.

Fig. 2 shows the cyclic voltammograms (CVs) of different electrodes in 0.1 M PBS (pH=7.0) at a scan rate of 50 mV s^{-1}. It can be seen that no redox peaks are observed at the GCE (curve a), DHs/GCE (curve b) and C-Dots/LDHs/GCE (curve d). However, a pair of redox peaks appears at HRP/LDHs/GCE and HRP/C-Dots/LDHs/GCE (curves c and e) which should be ascribed to the direct electrochemistry of HRP (Li et al., 2012). Moreover the peak currents at HRP/C-Dots/LDHs/GCE are higher than that at HRP/LDHs/GCE. The result indicates that although HRP can realize direct electrochemistry when immobilized by CoFe-LDHs, the

addition of C-Dots can further accelerate the electron transfer reaction of HRP. The role of C-Dots may be similar to that of carbon (C) in HRP/Ag@C/ITO (Guo et al., 2013) and ionic liquid (IL) in enzyme/GR-IL/GCE (Gholivand and Khodadadian, 2014) which provides a desirable microenvironment for enzyme to transfer electrons to the surface of the electrode.

To uncover the process of the direct electrochemistry of HRP on HRP/C-Dots/LDHs/GCE, the effect of scan rate on the response of this electrode is investigated and the results are presented in supplementary information (Fig. S1). As shown in Fig. S1, both cathodic and anodic peak currents (I_{pc} and I_{pa}) increase linearly with the scan rate (υ), which implies that the electrode reaction is a surface-controlled redox process. Furthermore the ratio of I_{pc}/I_{pa} is close to 1 at all studied scan rates, indicating that the electrochemical reaction of HRP on this electrode is quasi-reversible.

The electron transfer rate constant (k_s) of HRP on the modified electrode can be estimated according to Lavorin's theory (Laviron, 1979). In our case (Fig. S1A) the peak to peak separation (ΔE_p) is smaller than $200/n$ mV, so k_s should be calculated from the following equation:

$$k_s = mnF\upsilon/RT$$

where F, R and T have their usual significance, n is the electron transfer number, υ is the scan rate, and m is a parameter which can be determined according to the literature (Laviron, 1979). As the scan rate is 500 mV s^{-1}, the electron transfer coefficient α is taken as 0.5, $1/m$ is obtained as 2.3, and k_s is estimated as 8.46 s^{-1}. The k_s value obtained here is higher than that reported at other HRP-based modified electrodes, suggesting that C-Dots/LDHs hybrid provides a good environment to facilitate the electron transfer of HRP (Kong et al., 2003; Mahboubeh et al., 2012; Hong et al., 2007).

It has been reported that the electrochemical property of HRP is pH dependent (Guo et al., 2013). Herein the effect of pH on the HRP/C-Dots/CoFeLDHs/GC electrode is investigated and their CVs are recorded in Fig. S2A. It can be seen that the peak currents vary with pH and reach the optimal value at pH 7.0 which is the right physiological pH and suitable for enzymes to exist stably. From a practical point of view, pH 7.0 would be selected for further investigations.

On the other hand, both anodic and cathodic peak potentials shift negatively with the pH from 5.0 to 9.0 (Fig. S2B). The apparent formal peak potentials ($E^{\circ\prime}$) show a linear correlation with pH and the slope is −51.8 mV/pH which is close to the theoretical value for a reversible, one-proton coupled with one-electron redox reaction process at 298.15 K (Wu et al., 2008).

3.3. Electrocatalytic performance of HRP/C-Dots/LDHs/GCE toward H_2O_2

The electrocatalytic performances of the HRP/C-Dots/LDHs/GCE toward H_2O_2 were studied by cyclic voltammetry (CV). As shown in Fig. 3, with the addition of H_2O_2, the cathodic peak current increases and the anodic peak current decreases, indicating that the HRP/C-Dots/LDHs/GCE has a remarkable electrocatalytic activity towards H_2O_2 reduction.

The analytical performance of the HRP/C-Dots/LDHs/GCE toward H_2O_2 was also evaluated by chronoamperometry at a potential of −0.35 V. Fig. 4 shows the chronoamperometric responses and the corresponding calibration curves for the biosensor toward H_2O_2. The current response is proportional to the concentration of H_2O_2 in the range of 0.1–23.1 μM with a good correlation coefficient (R^2=0.9942). The detection limit (LOD) is estimated to be 0.04 μM at a signal-to-noise of 3.

It should be pointed that LDHs/GC and C-Dots/LDHs/GC modified electrodes have no electrochemical activity under tested conditions in this work (as shown in Fig. 2). However, it has been reported that both CoFe-LDHs (Zhang et al., 2012) and C-Dots (Shi et al., 2011) possess peroxidase activity and they can catalytically oxidize 3.3′,5.5′-tetramethylbenzidine (TMB) by H_2O_2. Can they also show catalytic activity toward H_2O_2 electro-reduction? To answer this, the amperometric responses of H_2O_2 at C-Dots/GCE, CoFeLDHs/GCE, and C-Dots/LDHs/GCE were also investigated as presented in Figs. S3–S5. The results show that the studied

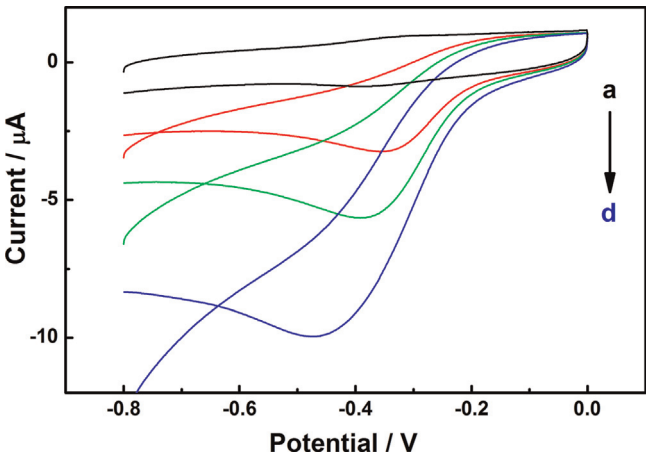

Fig. 3. CVs of the HRP/C-Dots/LDHs/GCE in 0.1 M pH 7.0 PBS solution in (a) 0 nM, (b) 2.0 mM, (c) 4.0 mM, and (d) 8.0 mM H_2O_2 at a scan rate of 50 mV s^{-1}.

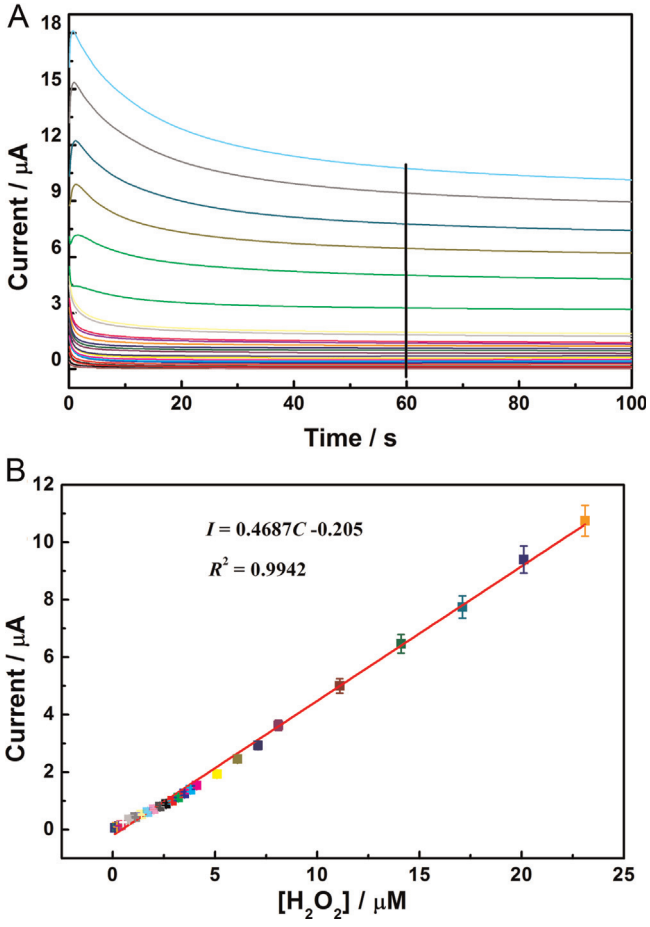

Fig. 4. (A) The amperometric response of the HRP/C-Dots/LDHs/GCE to different concentrations of H_2O_2 from 0.1 μM to 23.1 μM in 0.1 M pH 7.0 PBS. The applied potential was −0.35 V. (B) The corresponding calibration curve of $I-C$ obtained by chronoamperometry.

Table 1
Comparison of analytical performances of HRP/C-Dots/LDHs/GCE electrode with other biosensors.

Electrode material	Linear range (μM)	LOD (μM)	Sensitivity (mA mM^{-1} cm^{-1})	Reference
C-Dots/GCE	1.0–3.5	0.55	0.055	This work
LDHs/GCE	1.0–6.0	0.68	0.061	This work
C-Dots/LDHs/GCE	0.5–7.5	0.46	0.12	This work
HRP/C-Dots/LDHs/GCE	0.1–23.1	0.04	0.47	This work
HRP-Ag@C/ITO	0.5–140	0.2	–	Mao et al. (2013)
HRP/RTIL/GNPs-TNTs/Nafion	5.0–1000	2.1	–	Liu et al. (2012)
Nafion/HRP/Zr-IP6/GCE	0.667–6.0	0.53	–	Dong et al. (2010)
Gold-nanoparticle-adsorbed poly (thionine) film	5–150	1.5	–	Ahammad et al. (2011)

electrodes all have concentration-relative electrochemical response toward H_2O_2 and comparison of analytical performances toward H_2O_2 is summarized in Table 1. It could be seen that HRP/C-Dots/LDHs/GCE shows optimal analytical performances among the studied electrodes in this work from the point of view of linear range, LOD, or sensitivity. Especially for the sensitivity, the value of C-Dots/LDHs/GCE is higher than that of both C-Dots/GCE and LDHs/GCE. With the addition of HRP, the sensitivity of HRP/C-Dots/LDHs/GCE is superior to that of C-Dots/LDHs/GCE which demonstrates the advantage of composite material in constructing electrochemical biosensors. The excellent analytical performance of HRP/C-Dots/LDHs/GCE can be attributed to the synergistic effect of HRP, C-Dots and CoFe-LDHs.

In addition, the analytical performances of reported HRP-based H_2O_2 biosensors are also listed in Table 1 and the HRP/C-Dots/LDHs/GCE are comparable to them.

3.4. Selectivity of HRP/C-Dots/LDHs/GCE

The selectivity of HRP/C-Dots/LDHs/GCE for H_2O_2 was investigated by adding some common interfering substances such as uric acid (UA), ascorbic acid (AA) and epinephrine (EP). The amperometric responses of chosen interferents with H_2O_2 at −0.35 V in 0.1 M pH 7.0 PBS are shown in Fig. 5. It can be seen that the interfering substances do not cause any significant change in the current response even when the concentration of the interfering substances is 10 times greater as that of H_2O_2. The result shows that the HRP/C-Dots/LDHs/GCE is highly selective to H_2O_2 determination under the applied experimental conditions.

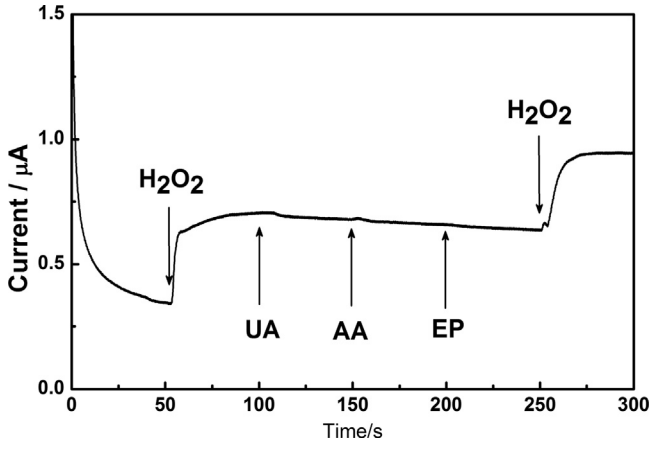

Fig. 5. The amperometric response of the HRP/C-Dots/LDHs/GCE to 10 mM UA, 10 mM AA, 10 mM E_p, and 1.0 mM H_2O_2 in 0.1 M pH 7.0 PBS. The applied potential was fixed at −0.35 V.

3.5. Reproducibility and stability of HRP/C-Dots/LDHs/GCE biosensor

The repeatability of the biosensor was first evaluated through its current response to 15 μM H_2O_2 for 10 successive determinations and the relative standard deviation (RSD) was found to be 4.5%. In addition, five HRP/C-Dots/LDHs/GC electrodes prepared independently were used to test 15 μM H_2O_2 and a RSD of 2.6% was obtained. The stability of this biosensor was investigated by measuring its response to 15 μM H_2O_2 after one week of storage at 4 °C in a refrigerator and the biosensor retained about 87.6% of its original response, indicating that the biosensor has good storage stability. However, after 14 days, the reduction current retained only about 43% of its initial value. The results show that the long-term stability of this biosensor is not satisfactory which can be overcome by fresh preparation as used.

4. Conclusions

The biosensor presented in this work was prepared by simply mixing CoFe-LDHs, C-Dots and HRP in order. The biosensor demonstrated fast direct electronic transfer behavior and good electrocatalytic reduction toward H_2O_2 due to the synergistic effect among CoFe-LDHs, C-Dots and HRP. The analytic performance of this biosensor was satisfactory due to its low detection limit and high selectivity. Considering its good analytical performance and easy use, the HRP/C-Dots/LDHs/GCE holds great promise for real H_2O_2 analysis in environment, food and industrial applications. In addition, the C-Dots/CoFe-LDH hybrid also provides a new platform for the construction of biosensors.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.08.054.

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