

Supramolecular assembly of enzyme on functionalized graphene for electrochemical biosensing

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ABSTRACT

The self-assembly of cyclodextrin (CD) functionalized graphene (GR) and adamantane-modified horseradish peroxidase (HRP-ADA) by host–guest supramolecular interaction into novel nanostructures in aqueous solution is reported in the present study. Electrochemical impedance spectroscopy and cyclic voltammetry were applied to characterize the self-assembly process and study the electrochemical behaviors of the immobilized proteins. UV–vis spectra indicated that the native structure of HRP was maintained after the assembly, implying good biocompatibility of CD-functionalized GR (CD-GR). Furthermore, the HRP-ADA/CD-GR composites were utilized for the fabrication of enzyme electrodes (HRP-ADA/CD-GR electrodes). The proposed biosensor showed good reproducibility and high sensitivity to H_2O_2 with the detection limit of 0.1 μM . In the range of 0.7–35 μM , the catalytic reduction current of H_2O_2 was proportional to H_2O_2 concentration.

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1. Introduction

GR, as a two-dimensional nanomaterial of sp^2 -bonded carbon, has attracted increasing interest since its discovery in 2004. Because of its novel properties, such as exceptional thermal and mechanical properties, high electrical conductivity, GR sheets have been extensively studied in synthesizing nanocomposites (Stankovich et al., 2006; Xu et al., 2008; Muszynski et al., 2008; Williaris et al., 2008) and fabricating various microelectrical devices, such as battery (Cassagneau and Fendler, 1998), field-effect transistors (Gilje et al., 2007), ultrasensitive sensors (Schedin et al., 2007), and electromechanical resonators (Bunch et al., 2007). At present, many methods have been developed to produce GR, including mechanical exfoliation, chemical vapor deposition, and chemical or thermal reduction of graphite oxide. Among them, chemical conversion from graphite oxide is attractive for its low-cost and massive scalability (Rao et al., 2010; Wang et al., 2009). However, high-quality GRs with high hydrophobic property, unless well separated from each other, tend to form irreversible agglomerates or even restack to form graphite through strong π – π stacking and Van der Waals interaction (D. Li et al., 2008). The prevention of aggregation is of particular

importance for GR because most of their unique properties are only associated with individual sheets. Some methods containing covalent and noncovalent functionalization of GR have been used for enhancing its solubility and self-assembly properties (Liu et al., 2009; X.L. Li et al., 2008). The latter is particularly promising, since the GR functionalized through noncovalent modification can retain its electronic structure (Tu et al., 2010).

CDs are cyclic oligosaccharides consisting of six, seven or eight glucose units called α -, β - and γ -CDs. These CDs are toroidal in shape with a hydrophobic inner cavity and a hydrophilic outer side. The unique structural properties can make them bind a number of inorganic, organic and biological molecules into their cavities to form stable host–guest inclusion complexes with high molecular selectivity (Wang and Khaledi, 1996; Reeman et al., 2009). In addition, CDs are environmentally friendly, water-soluble, and can increase the solubility and dispersibility of functional materials. Once GR is functionalized with CDs, it is likely to obtain new materials simultaneously possessing the unique properties of GR (large surface area and high conductivity) and CDs (high supramolecular recognition and enrichment capability) through combining their individual characteristics. Therefore, the integration of GR and CDs will provide potential applications in the fields of electrocatalysis, luminescence, and biosensors (Tan et al., 2010; Guo et al., 2010; Ogoshi et al., 2010).

In the present study, we for the first time propose a simple method to assemble GR with enzymes into novel nanocomposites

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via host–guest supramolecular interactions for the fabrication of enzyme electrodes, as illustrated in Scheme 1. CD-GR nanocomposite was prepared via a simple wet-chemical strategy. The CD-GRs were well-dispersed in aqueous solution, which was confirmed by the transmission electron microscope (TEM). And then, by host–guest supramolecular interactions between HRP-ADA and the CD-GR, HRP self-assembled with CD-GR on the surface of a glassy carbon (GC) electrode to form an HRP-ADA/CD-GR/GC electrode. The experimental results revealed that such an electrochemical platform not only preserved the native structure of the immobilized enzyme but also exhibited good electron-transfer properties for HRP. Meanwhile, the as-prepared HRP-ADA/CD-GR/GC electrode exhibited good analytical performance toward the quantification of H_2O_2 , with a wide linear range, good reproducibility, and long-term stability.

2. Experimental

2.1. Apparatus and reagents

TEM image was taken with a JEM-3010 transmission electron microscope (JEOL Co., Ltd., Japan). The UV–vis spectra were recorded on a Multispec-1501 Shimadzu Hyper UV–vis spectrophotometer. The cyclic voltammetric, amperometric and electrochemical impedance spectroscopy measurements were carried out on a CHI 760B electrochemical workstation (Shanghai, China). Electrochemical impedance spectroscopy was performed in 5 mM $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (1:1) mixture with 0.1 M KCl at the formal potential of the 240 mV using alternating voltage of 5 mV. The frequency range was from 1 Hz to 100 kHz. A three-electrode cell (10 mL) was used with the modified glassy carbon (GC) electrode as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum foil electrode as the counter electrode. All potentials were measured versus the SCE, and all experiments were carried out at room temperature.

Horseradish peroxidase (HRP; molecular weight = 44 kDa) was purchased from Sigma Chemical (USA). Graphite powder (99.95%, 325 mesh), β -cyclodextrin, hydrogen peroxide solution (30 wt% aqueous), hydrazine solution (50 wt%) and N-(3-dimethylaminopropyl)-N'-tylcarbodiimide (EDAC) were purchased from the Beijing Chemical Reagent factory (Beijing, China) and used as received. All other reagents were of analytical grade and double distilled water was used throughout the experiments.

2.2. Synthesis of CD-GR nanocomposite and pure GR

Graphene oxide nanosheets were synthesized from natural graphite by a modified Hummers' method (Kovtyukhova et al.,

1999). A CD-GR nanocomposite was prepared as follows (Guo et al., 2010): a 20.0 mL portion of the homogeneous graphene oxide dispersion (0.5 mg mL^{-1}) was mixed with 20.0 mL of 80 mg mL^{-1} β -CD aqueous solution and 300.0 μL of ammonia solution, followed by the addition of 20 μL of hydrazine solution. After being vigorously shaken or stirred for a few minutes, the mixture was then heated to reflux at 100°C for 4 h to prepare the CD-GR nanocomposite. After cooling to room temperature, the resulting materials were then centrifuged and washed three times with distilled water to remove excess hydrazine and β -CD. Additionally, the preparation of pure GR was similar with CD-GR nanocomposite except there was no addition of CD.

2.3. Preparation of the HRP-ADA/CD-GR/GC electrode

HRP was first conjugated with 1-adamantane carboxylic acid via a carbodiimide-catalyzed reaction as previously described (Villalonga et al., 2007). A reaction mixture composed of HRP (20 mg), 1-adamantane carboxylic acid (100 mg), and EDAC (50 mg) in deoxygenated 50 mM potassium phosphate buffer pH 6 (5 mL) was stirred overnight at 4°C . The solution was dialyzed against phosphate buffer pH 6 several times and kept at 4°C when not used for measurements.

To prepare the enzyme-modified electrode, a bare GC electrode was polished to a mirror-like with alumina powder (0.3 mm), rinsed thoroughly with double distilled water, then washed successively with double distilled water, anhydrous ethanol and acetone in an ultrasonic bath, and dried under N_2 before use. In order to accomplish electrode coating, 5 μL -aliquots of the CD-GR solution (0.5 mg mL^{-1}) were successively deposited and dried on the electrode surface. The CD-GR modified electrode was then immersed in 0.1 M phosphate buffer solution (pH 7.0) containing HRP-ADA (4 mg mL^{-1}) for 4 h to construct the HRP-ADA/CD-GR/GC electrode.

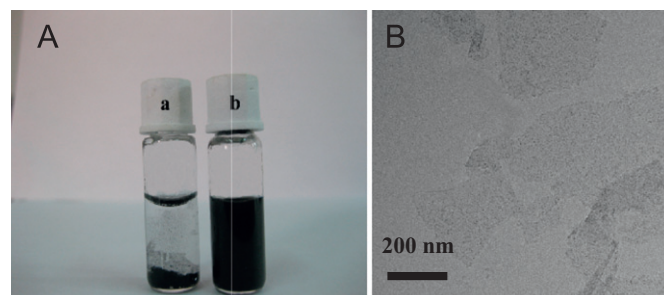
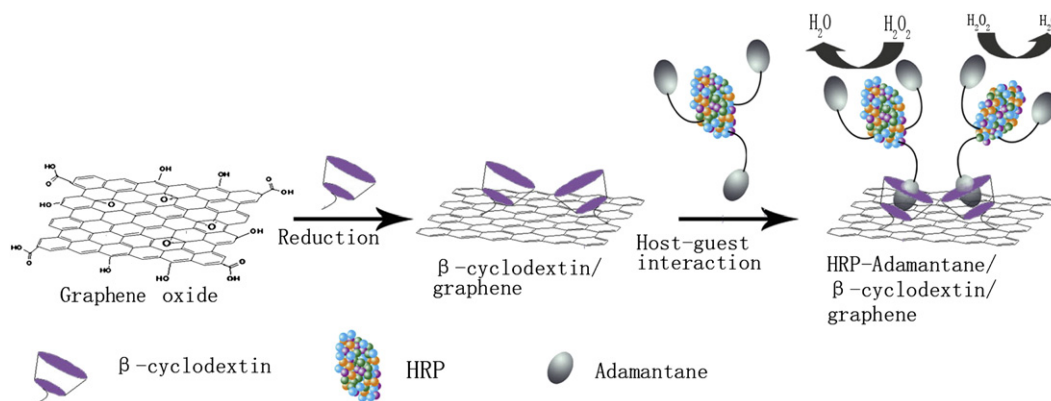


Fig. 1. (A) Photographic images of unmodified GR (a) and CD-GR (b) in water (2 mg mL^{-1}); (B) TEM image of CD-GR.



Scheme 1. Schematic representation of fabrication of the HRP-ADA/CD-GR/GC electrode and the principle for H_2O_2 determination.

The as-prepared HRP-ADA/CD-GR/GC electrode was rinsed with deionized water and stored at 4 °C in a refrigerator when not in use.

3. Results and discussion

3.1. Characterization of the CD-GR nanocomposite

GR chemically derived from graphite oxide cannot be well-dispersed in aqueous solution due to its hydrophobic nature, so it always forms agglomerates with badly ordered architectures. As shown in Fig. 1A(a), GR agglomerates are completely settled down at the bottom of the vial from aqueous solution immediately after removal of the sonication probe, thus leaving the supernatant colorless. By contrast, the CD-GR suspension even at a concentration as high as 2 mg mL⁻¹ appears to be very homogenous and stable (no sediments were observed for at least 2 months) (Fig. 1A(b)). TEM image of the CD-GR, as shown in Fig. 1B, clearly illustrates the transparent and flake-like shapes (Zeng et al., 2010).

3.2. UV-vis spectroscopic analysis of the HRP-ADA/CD-GR composite

UV-vis absorbance spectroscopy is usually employed to characterize the conformational change of protein and the interaction between protein and other composition (Zhang et al., 2010). As shown in Fig. 2, the absorption peak of CD-GR (curve a) appeared at 272 nm, which is consistent with that reported in literature (Vadukumpully et al., 2009). It is evident that a peak appeared at 403 nm in the spectrum of the HRP-ADA (curve c). The Soret band of HRP (curve b) at 403 nm was unaffected during the conjugation process, which suggests that the conjugated HRP maintained its native structure. The HRP-ADA further interacted with CD-GR through host-guest chemistry. The absorption band at 403 nm has no shift except the erect movement of the whole curve (curve d), which indicated that the interaction between the HRP and GR did not destroy the natural structure or change the fundamental microenvironment of HRP.

3.3. Electrochemical impedance spectroscopy (EIS) characterization of self-assembly process

In electrochemical impedance spectroscopy measurements, the semicircle diameter of impedance equals the electron transfer

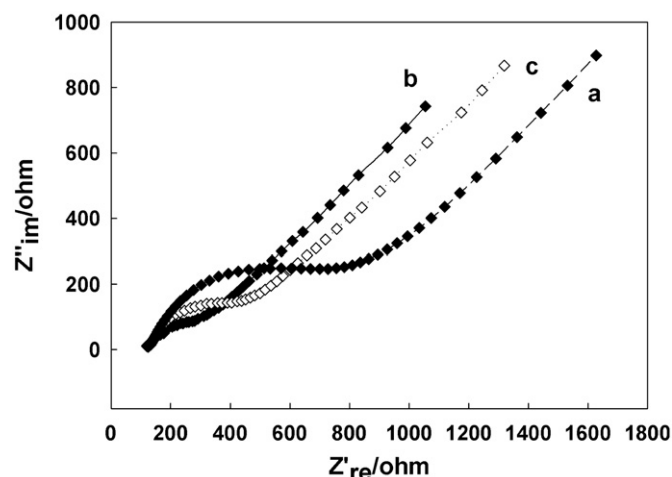


Fig. 3. The representative impedance spectrum of the bare GC electrode (a), CD-GR/GC electrode (b) and HRP-ADA/CD-GR/GC electrode (c) in 5.0 mM K₃Fe(CN)₆/K₄Fe(CN)₆ (1:1) containing 0.1 M KCl.

resistance (Ret), which controls the electron transfer kinetics of the redox probe at the electrode interface and is an important parameter. Fig. 3 presents the representative impedance spectrum of the bare GC electrode (a), CD-GR/GC electrode (b) and HRP-ADA/CD-GR/GC electrode (c) in 5.0 mM K₃Fe(CN)₆/K₄Fe(CN)₆ (1:1) containing 0.1 M KCl. When CD-GR was modified into the GC electrode (curve b), the semicircle decreased distinctly compared with the bare GC electrode (curve a), indicating that CD-GR could accelerate the electron transfer between the electrochemical probe [Fe(CN)₆]^{3-/4-} and the GC electrode, which is attributed to the significantly improved electrical conductivity of CD-GR films. After HRP-ADA assembled on the CD-GR/GC electrode, the semicircle dramatically increases (curve c), indicating that the presence of the HRP molecules on the electrode surface blocked the electron transfer.

3.4. Electrochemical properties of HRP-ADA/CD-GR/GC electrode

The electrocatalytic properties of the HRP-ADA/CD-GR/GC electrode toward H₂O₂ reduction were investigated by cyclic voltammetry. As shown in Fig. 4A, at the HRP-ADA/CD-GR/GC electrode, the reduction (cathodic) peak current at around -0.15 V increases significantly with increase of H₂O₂ concentration, accompanied by decrease of the oxidation peak current, demonstrating a typical electrocatalytic reduction process of H₂O₂. The mechanism for the whole electrode response process could be expressed as the following reactions (Li et al., 2010):

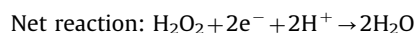
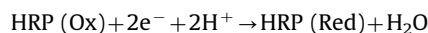
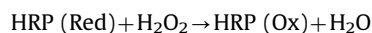


Fig. 4B shows the cyclic voltammograms of the HRP-ADA/CD-GR/GC electrode recorded in 0.1 M PBS solution at different scan rates. It is found that both the anodic and cathodic peak currents clearly increase with increasing potential scan rate. Moreover, the redox peak currents are proportional to the scan rate, ν (inset of Fig. 4B). The linear regression equations and correlation coefficients of the oxidation peak current (I_{pa}) and reduction peak current (I_{pc}) of the scan rates are expressed as $I_{\text{pa}} (\mu\text{A}) = 0.37904 + 0.11219\nu (\text{mV s}^{-1})$ and $I_{\text{pc}} (\mu\text{A}) = -3.9523 - 0.1295\nu (\text{mV s}^{-1})$ with the correlation coefficients (R^2) of 0.99972 and 0.99262, respectively. It indicates

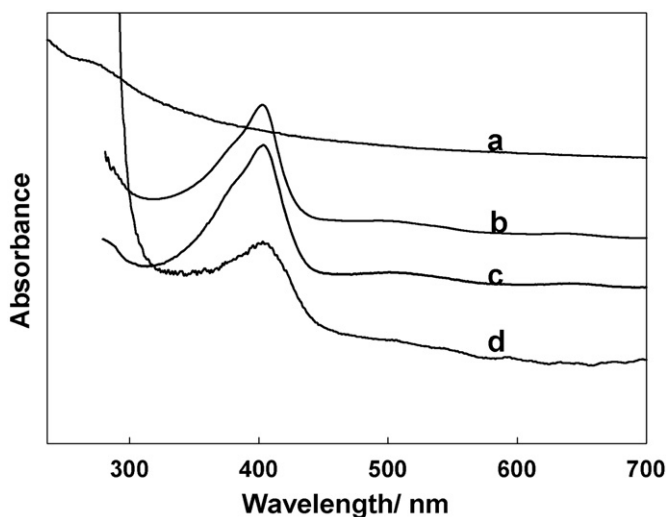


Fig. 2. UV-vis absorption spectra of (a) CD-GR, (b) HRP, (c) HRP-ADA and (d) HRP-ADA/CD-GR in PBS at pH 7.0.

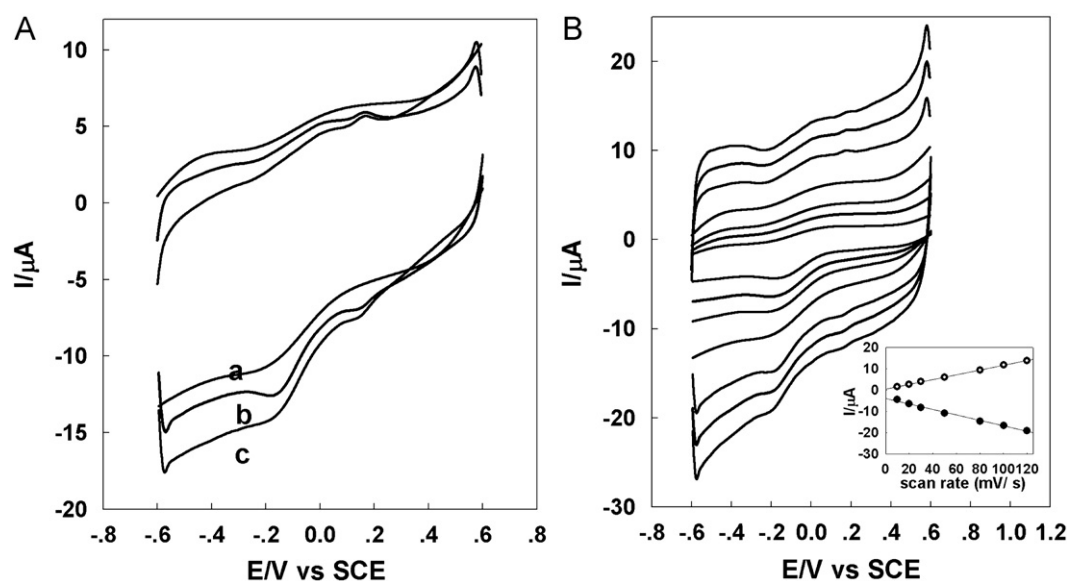


Fig. 4. (A) Cyclic voltammograms of HRP-ADA/CD-GR/GC electrode in the absence (a) and presence of 1.0×10^{-4} M (b), 3.0×10^{-4} M (c) H_2O_2 in 0.1 M PBS (pH 7.0). Scan rate: 50 mV s^{-1} . (B) Cyclic voltammograms of the HRP-ADA/CD-GR/GC electrode recorded in 0.1 M PBS solution at different scan rates (inner to outer): 10, 20, 30, 50, 80, 100 and 120 mV s^{-1} , respectively. Inset: plots of oxidation peak current and reduction peak current versus the scan rate.

that the surface reaction of HRP-ADA/CD-GR/GC electrode is a typical surface-controlled electrochemical process.

3.5. Optimization of detection variables

The pH value is one of the parameters that affect the response of HRP-ADA/CD-GR/GC electrode to H_2O_2 . Fig. S1A presents the pH dependence of the amperometric response of $20 \mu\text{M}$ H_2O_2 in the pH range 5.0–9.0 at the potential of -0.15 V . It can be seen that the current increased as the pH changed from 5.0 to 7.0 and then decreased above pH 7.0. The maximum response was obtained at pH 7.0, which was consistent with the previously reported HRP-based modified electrode (Okuma and Watanabe, 2002). This indicated that the immobilization procedure did not alter the inherent properties of HRP. Therefore, pH 7.0 PBS was used as the electrolyte in subsequent experiments.

Fig. S1B displays the dependence of applied potential on the amperometric response of the biosensor to $20 \mu\text{M}$ H_2O_2 in pH 7.0 PBS. When the applied potential was changed from 0 to -0.35 V , the maximum response current was observed at -0.15 V . Taking the sensitivity and the signal/noise ratio into consideration, -0.15 V was chosen as the optimum applied potential.

3.6. Amperometric sensing of H_2O_2

The amperometric response of the HRP-ADA/CD-GR/GC electrode to successive additions of H_2O_2 was further evaluated under the optimized experimental conditions. Fig. 5 shows the typical current–time dynamic response of the HRP-ADA/CD-GR/GC electrode toward H_2O_2 . The electrode responded quickly to the change of H_2O_2 concentration and reaches about 95% of the steady-state current within 4 s, which was faster than those reported for HRP-Methylene blue/MWNT electrode (30 s) (Xu et al., 2003), Hb/ZrO₂/chitosan electrode (10 s) (Zhao et al., 2005), and HRP/Au/TiO₂/Ti electrode (5 s) (Kafi et al., 2008). The amperometric signal shows linear correlation to H_2O_2 concentration in the range from 0.7 to $35 \mu\text{M}$ with a correlation coefficient of 0.998, which covers three orders of magnitude of H_2O_2 concentrations. It was much wider than that of Hb/ZnO-MWCNTs/naftion

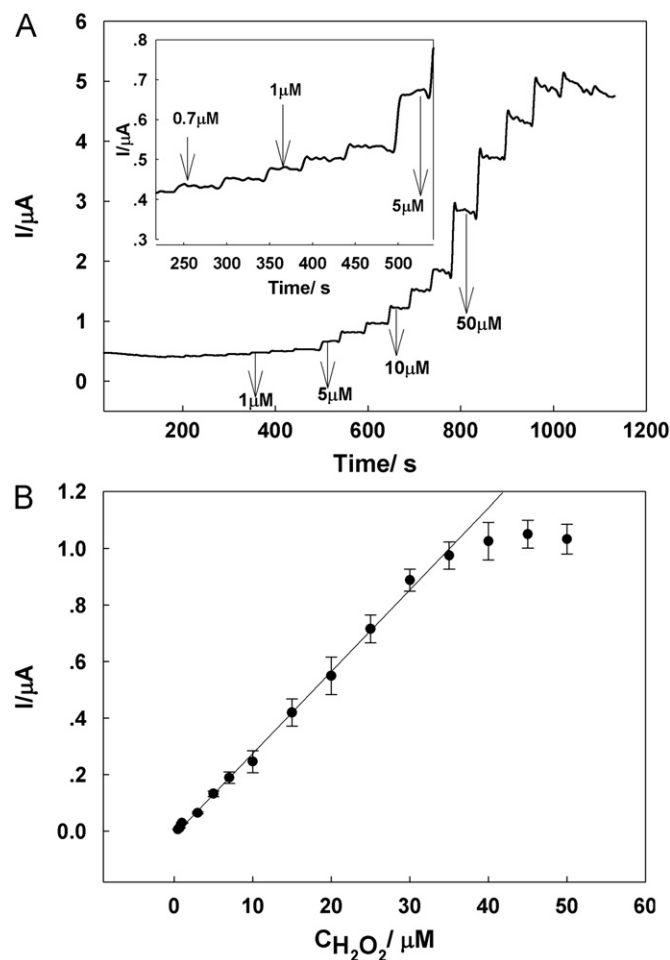


Fig. 5. (A) Typical current–time dynamic response of the HRP-ADA/CD-GR/GC electrode to successive addition of different concentrations of H_2O_2 in 0.1 M PBS solution (pH 7.0) at the working potential of -0.15 V ; Inset: amplified response curve; (B) the corresponding calibration plot of amperometric response toward H_2O_2 .

electrode (1.5–30 μM) (Liu et al., 2004) and the HRP/TiO₂ nanorod electrode (0.8–35 μM) (Liu et al., 2010). The detection limit was estimated to be 0.1 μM (based on $S/N=3$) for H₂O₂, which was lower than that of 123 μM at the HRP/NiO nanoparticles electrode (Zhou et al., 2010), 9 μM at the HRP/ZnO/Au nanoparticles/naion electrode (Xiang et al., 2009) and 1.7 μM at the HRP/Au/graphene/chitosan electrode (Mohammadi et al., 2009). The sensitivity of the biosensor estimated was 783.4 $\text{mA M}^{-1} \text{cm}^{-2}$. This value was also higher than those reported in the literatures (e.g. 109 $\text{mA M}^{-1} \text{cm}^{-2}$ at HRP-ADA on CD-SH coated Au electrodes (Camachoa et al., 2009) and 356.6 $\text{mA M}^{-1} \text{cm}^{-2}$ at the HRP/ss-DNA/graphene/GC electrode (Zhang et al., 2010).

Based on the Lineweaver–Burk equation (Kamin and Willson, 1980), the apparent Michealis–Menten constant (K_m^{app}) is calculated to be 0.18 mM for HRP-ADA/CD-GR/GC electrode, which was much smaller than some of the previously reported values with other electrodes (23.85 mM for the HRP/sol-gel-derived ceramic carbon nanotube film-modified GC electrode (Chen and Dong, 2007), 5.5 mM for the HPR-modified electrode (Ferri et al., 1998) and 0.684 mM for the HRP/graphene oxide/naion-modified electrode (Zhang et al., 2012)). Such a low K_m^{app} value for HRP-ADA/CD-GR/GC electrode shows that the enzyme intercalated in GRs possesses high catalytic efficiency to the reduction of H₂O₂ with a low diffusion barrier.

3.7. Selectivity, reproducibility and stability of the biosensor

The interferential experiment of this biosensor was performed by comparing the amperometric response of 20 μM H₂O₂ before and after adding 200 μM ; some possible interferents into 0.1 M pH 7.0 PBS and the results were given in Table S1. As shown, glucose, ethanol, oxalic acid, ascorbic acid and uric acid did not interfere with the determination of H₂O₂. The good selectivity of this biosensor is largely attributed to the low working potential (-0.15 V).

The reproducibility and repeatability of the developed biosensor were examined. In 10 different electrodes constructed independently by the same procedure, a relative standard deviation (RSD) of 4.5% was obtained toward 10 μM H₂O₂, indicating the reliability of the method. A set of 10 different amperometric measurements for 10 μM H₂O₂ with a single electrode yielded a RSD of 3.6%.

The stability of the biosensor was explored. When not in use, the proposed biosensor was stored at 4 °C in a refrigerator. The response to 10 μM H₂O₂ at -0.15 V was tested each week. The data show that the sensitivity of the electrode remained relatively constant over the first 14 days. After 35 days of storage, the response of the sensor only decreased 3.7% compared to the initial response, which shows long-term stability.

3.8. Real sample analysis

The practical applications of the designed biosensor were evaluated by determination of recovery of H₂O₂ in real wastewater samples. The analytical results are shown in Table S2. One observed that the results obtained in real water samples showed good results with average recoveries from 96.5% to 106.0%, which confirmed that the proposed biosensor was applicable for practical H₂O₂ detection.

4. Conclusion

A novel HRP-ADA/CD-GR/GC electrode was constructed through supramolecular self-assembly between HRP-ADA and CD-GR nanocomposites on the surface of a GC electrode. The UV–vis spectral

results showed that HRP was immobilized onto the CD-GR nanocomposite without denaturation. The HRP-ADA/CD-GR/GC electrode performed excellent electrocatalytic performance toward the reduction of H₂O₂ with fast response, wide linear range and good stability. The good performance of the proposed biosensor and good recoveries for determination of H₂O₂ in real sample analysis will benefit the further implementation of this technique in both biological and environmental applications, and the developed strategy of tethering enzymes into GR may pave the way in developing new solid support–enzyme nanohybrids for the fabrication of biosensors.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2013.01.065>.

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