Horseradish peroxidase-catalyzed synthesis of poly(thiophene-3-boronic acid) biocomposites for mono-/bi-enzyme immobilization and amperometric biosensing

Yi Huang, Wen Wang, Zou Li, Xiaoli Qin, Lijuan Bu, Zhiyong Tang, Yingchun Fu, Ming Ma, Qingji Xie, Shouzhuo Yao, Jiming Hu

1. Introduction

Boronic acid compounds have attracted much attention in both chemistry and biology. The boronic acid group can form chemical bonds with 1, 2-, or 1, 3-diol groups to generate five or six-membered cyclic complexes at appropriate pH, which has been widely used as the recognition motif for chemo/biosensing (Park et al., 2008; Rick and Chou, 2008; Takahashi et al., 2008), enrichment and separation of biomolecules (Lin et al., 2011), and supermolecular assembly (Bapat et al., 2011). Most of proteins are glycoproteins, e.g., horseradish peroxidase (HRP) has a glycosylation degree of about 16.8–21 wt% (Aibara et al., 1982), and glucose oxidase (GOx) of about 16–25 wt% (Courjean et al., 2009). Recently, the immobilization of glycoprotein enzyme for biotechnology application based on boronic acid-diol affinity has attracted much attention (Cui et al., 2011; Villalonga et al., 2011; Zayats et al., 2002). Glycoprotein antibody (Lin et al., 2009; Moreno-Guzmán et al., 2012) was also immobilized orientedly on a layer of boronic acid self-assembly for improved immunosensing. In contrast, reports on boronic acid group-bearing polymer for enzymatic biosensing applications are rather limited to date (Cui et al., 2011; Liu et al., 2006; Villalonga et al., 2011). Our group realized the entrapment of GOx into boronic acid based polymer matrix by chemical polymerization for high-performance biosensors and biofuel cells (Huang et al., 2012), which encourages us to explore enzymatic polymerization of the boronic acid monomers for enzyme immobilization and biosensing.

Chemical (Deore et al., 2008), electrochemical (Shoji and Freund, 2001), or enzymatic (Kobayashi and Makino, 2009) oxidation of monomers to synthesize polymers has been well established. Chemical polymerization is sometimes not suitable for immobilization of biomacromolecules owing to their denaturation under harsh reaction conditions (e.g., extreme pH, high temperature, strong oxidants, and...
bio-incompatible solvents). The electrochemical methods carry out only on the electrode surface and a large amount of production is limited. Recently, the enzymatic polymerization reaction as a viable environmentally friendly alternative has come to occupy an important position in the field of polymer chemistry (Cholli et al., 2005; Kim et al., 2007; Kobayashi and Makino, 2009; Walde and Guo, 2011). Multiple oxidoreductases, including peroxidases (Caramyshev et al., 2005; Cruz-Silva et al., 2008; Liu et al., 1999; Nagarajan et al., 2008; Rumbau et al., 2007), and oxidases (Cui et al., 2007; Fu et al., 2009; Kausaite-Minkstimiene et al., 2010; Tan et al., 2010), have been used in biocatalyzed polymerization in an aqueous buffer. For instance, lactate oxidase (Cui et al., 2007), GOx (Kausaite-Minkstimiene et al., 2010; Kausaite and Musiakeviciene, 2009), bilirubin oxidase (Aizawa et al., 1990) and laccase (Tan et al., 2010) were adopted for polymerization. Our group has developed several enzyme biosensors based on enzymatic polymerization with enzyme immobilized at high load/activity (Fu et al., 2009; Tan et al., 2010).

Polystyrene (PSt) is a promising conducting polymer with wide application potential in thermoelectrics (Taggart et al., 2011), electrochromic displays (Nichol et al., 2004), corrosion protection (Ocampo et al., 2005), and sensors (Kuwahara et al., 2009). However, high oxidation potential and nonaqueous solvent are needed for PSt synthesis (DiCarmine et al., 2011), which limit its biochemical application. Enzymatic syntheses of PTh derivatives with high water-solubility have been studied in recent years (Nagarajan et al., 2008; Rumbau et al., 2007). As we are aware, enzymatic polymerization of a boronic acid group-bearing soluble monomer for enzymatic biosensing applications has not been reported to date.

Herein, a facile enzymatic polymerization protocol is suggested to prepare enzyme-containing polymeric biocomposites (PBCs) for high-performance mono-/bi-enzyme amperometric biosensing. The monoenzyme PBCs are produced by HRPCatalyzed polymerization of thiophene-3-boronic acid (TBA) monomer by addition of H2O2, and the bienzyme PBCs are prepared in aqueous solution containing HRP and GOx by either directly added or enzymatically generated H2O2.

The enzyme polymerization protocol provides mild procedures and biocompatible microenvironment for enzyme immobilization. The PBCs are dialysis-isolated and then cast-coated on Au electrodes to prepare mono-/bi-enzyme amperometric biosensors. Cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), chronocamperometry, and UV–vis spectrophotometry are used for material characterization and process monitoring. The thus-prepared biosensors exhibit high sensitivity, rapid response, good reproducibility, long-term stability and freedom from other potentially interfering species.

2. Experimental

2.1. Instrumentation and chemicals

The electrochemical experiments were performed on a CHI660C electrochemical workstation (CH Instruments Co., USA). A gold disk electrode (2 mm diameter) served as the working electrode, a KCl-saturated calomel electrode (SCE) as the reference electrode, and a carbon rod as the counter electrode. All potentials are cited versus SCE. UV–vis absorption spectra were collected on a UV2450 UV–vis spectrophotometer (Shimadzu Co., Japan). Scanning electron microscopy (SEM) images were collected on a JEM-6700F field emission scanning electron microscope.

HRP (EC 1.11.1.7, 300 U mg–1) was purchased from Roche. GOx (EC 1.1.3.4; type II from Aspergillus niger, 150 kU g–1) and 3,3',5,5'-tetroxymethylbenzidine (TMB) were obtained from Sigma. TBA was obtained from SooChiral Chemical Science and Technology Co. Ltd. Thiopene was purchased from Aladdin, which was purified by three-distillation prior to use. Chloroacetic acid and trisodium citrate were purchased from Shanghai Chemicals Station. A 30 wt% H2O2 solution was purchased from Shanghai Taopu Chemical Factory, and a fresh solution of H2O2 was prepared daily. Chitosan (CS, from crab shells, 90 wt% deacetylated) and glucose were purchased from Sinopharm Chemicals Co. Ltd. Ammonium persulfate (APS) and 98 wt% concentrated H2SO4 were obtained from Tianjin Damao Chemical Reagent Factory. Dialysis bag with a 8–14 kDa molecular weight cut-off was obtained from Beijing SolarBio Science and Technology Co. Ltd. Multiwalled carbon nanotubes (MWCNTs) material was obtained from Shenzhen Nanopart Co. Ltd. with 20–40 nm diameter and 5–15 μm length, and purified before use according to a previous report (Zhang et al., 2010). Gold nanoparticles (AuNPs) were prepared according to the citrate reduction protocol (Grabar et al., 1995). 10 mM phosphate buffer solution (PBS, 4 mM KH2PO4 + 6 mM Na2HPO4, pH 7.0) was used for enzymatic polymerization, and 0.1 M PBS (0.04 M KH2PO4 + 0.06 M Na2HPO4, pH 7.0) was also used as the supporting electrolyte in other experiments as specified. A 0.50 wt% CS solution was prepared in 10 M acetate buffer solution (pH 5.4). 1.00 M glucose stock solution was allowed to mutarotate overnight at room temperature before use. A 10 mM stock standard solution of TMB was prepared in DMSO. All other chemicals were of analytical grade or better quality and used as received. Milli-Q ultrapure water (Millipore, ≥ 18 MΩ cm) was used throughout. All experiments were performed at room temperature around 25 °C.

2.2. Enzymatic synthesis of various PBCs

The HRP–PTBA PBCs were synthesized as follows (Scheme 1). Into 2 mL of 10 mM stirring PBS containing 2 mg mL–1 HRP and 1.5 mg mL–1 TBA, 250 μL 0.3 wt% H2O2 was added dropwise (ca. 10 drops/min). The reaction mixture was stirred for 2 h, and then the unreacted monomer and oligomers were removed from solution by 12 h dialysis against pure water. Similarly, thiopene (1.5 mg mL–1) was used instead of TBA, and the resulting PBCs are denoted as HRP–PSt. In addition, HRP–PTBA–MWCNTs and HRP–PTBA–AuNPs bionanocomposites were prepared in reaction suspensions containing 0.2 mg mL–1 MWCNTs or ca. 1.5 nM AuNPs. For bienzyme PBCs (Scheme S1), and the reaction solution was 2 mL of 10 mM PBS containing 1 mg mL–1 GOx, 2 mg mL–1...
HRP and 1.5 mg mL\(^{-1}\) TBA. The enzymatic reactions were initiated by addition of 250 \(\mu\)L 0.3 wt% \(\text{H}_2\text{O}_2\) (Route I) or 250 \(\mu\)L 100 mM glucose (Route II), and the resulting PBCs are denoted as GOx–HRP–PTBA(\(\text{H}_2\text{O}_2\)) or GOx–HRP–PTBA(gluc), respectively.

2.3. Electrode modifications

As shown in Scheme 1, the modified electrodes were prepared via a simple cast-coating method. Firstly, to remove possible surface contamination, the bare Au electrode was cleaned by chemical and electrochemical rinsing according to a reported protocol (Zhang et al., 2007b), as briefly described in the supplemental information.

Then, gold was electroplated on the cleaned Au electrode to achieve a rough surface for improved sensitivity by multiple potential step electrolysis from 1.1 to 0 V with a pulse width of 0.25 s in 0.50 M aqueous \(\text{H}_2\text{SO}_4\) containing 2.0 mM HAuCl\(_4\) (Huang et al., 2012). The Au-plating time was optimized to be 300 s.

Subsequently, 2.5 \(\mu\)L of resulting PBCs mixture was cast onto the Au plate/Au electrode. After solvent evaporation, 1.5 \(\mu\)L of 0.50 wt% CS solution was cast-dried to prevent enzyme leaching and mechanically strengthen the enzyme film (CS/HRP–PTBA/Auplate/Au), and CS as a polysaccharide was selected here due to its excellent film-forming ability, high permeability, and good blocking effect for boronic acid residues (Huang et al., 2012). The enzyme electrode was rinsed with water several times before use. The bienzyme electrodes were fabricated similarly by using the bienzyme PBCs instead. For comparison, chemical polymerization of PTBA was synthesized using APS as the oxidant. Briefly, 2 mL of 100 mM fresh prepared APS aqueous solution was injected into 2 mL 4 mg mL\(^{-1}\) TBA aqueous solution, and stirred for 2 h. Then, the product was cleaned by repeated centrifugation till the supernatant became neutral. The PTBA precipitate was redispersed in 4 mL 10 mM PBS containing 2 mg mL\(^{-1}\) HRP, and 2.5 \(\mu\)L of the resulting composites and then 1.5 \(\mu\)L of 0.50 wt% CS solution were cast-coated on an Au electrode to form a CS/HRP/PTBA/Au plate/Au electrode. When not in use, the as-prepared enzyme electrodes were stored in PBS at 4 °C (refrigerator).

2.4. Measurements

PBCs samples for SEM characterization were prepared by cast-coating of each on a smooth silica sheet, followed by sputtering with a thin gold film.

CV experiments were performed in quiescent solution at a scan rate of 50 mV s\(^{-1}\), and the 1st cycle curves were adopted. For steady-state amperometric experiments, the working potential was set at –0.10 V in 0.1 M PBS containing 1 mM K\(_4\)Fe(CN)\(_6\), and the solution was stirred with a magnetic stirrer. For EIS experiments, 0.1 M PBS containing 0.1 M K\(_2\)SO\(_4\), 1 mM K\(_3\)Fe(CN)\(_6\), and 1 mM K\(_4\)Fe(CN)\(_6\) was used, and the parameters are detailed in Fig. S1.

UV–vis spectrophotometry was performed for visual inspection of the oxidation/polymerization reaction and evaluation of the enzymatic specific activity (ESA) of HRP. For ESA quantification, into a stirred colorimetric system of 3 mL 0.1 M citrate–phosphate buffer (pH 4.0) containing 0.1 mM TMB and 1 mM \(\text{H}_2\text{O}_2\), at native HRP was added to trigger the colorimetric reaction. After reacted for 1 min, 5 mL 0.5 M \(\text{H}_2\text{SO}_4\) was immediately added to terminate the enzyme reaction. Finally, absorbance at 450 nm was recorded (Cao et al., 2012). For enzymatic kinetics measurements, 3 mL colorimetric mixture as above was quiescent in 4 mL cuvette, and the absorbance at 450 nm towards time was then recorded after HRP addition.

3. Results and discussion

3.1. Synthesis and characterization of PBCs for mono-enzyme biosensing

As shown in Scheme 1, HRP acted as a biocatalyst for PTBA synthesis in the presence of oxidant \(\text{H}_2\text{O}_2\), similar to HRP-catalyzed
polymerization of 3,4-ethylenedioxythiophene also with H$_2$O$_2$ as the oxidant (Rumbau et al., 2007). HRP can be encapsulated in the resulting PBCs, since enzymatic polymerization should always take place around the enzyme molecules. As shown in Fig. 1A by digital image, oxidation and polymerization of TBA by H$_2$O$_2$ alone occurred very slowly, giving no visible color change. Addition of HRP suddenly changed the solution color to pink and gradually darkened the solution with time, indicating formation of PTBA. The successful encapsulation of hydrophilic HRP led to excellent dispersibility of the PBCs. UV-vis spectrophotometry (Fig. 1A) was also employed to monitor the oxidation and polymerization of TBA. The increase of peak absorbance at 293, 416 and 506 nm with time prove the progress of HRP-catalyzed synthesis of PTBA. Here, absorption peak at 293 nm indicates $\pi$-$\pi^*$ transition of 2,5-thiophenylene unit (Li et al., 2009); and the polaron absorption band near 416 and 506 nm are ascribed to $\pi$-$\pi^*$ transition of the quinoid 2,5-thiophenylene units, which are related to extension of the large $\pi$-conjugation along the molecular chain. The band around 240 nm is ascribed to the absorption of boronic acid residues and amino acid residues in HRP, and its increase with time may be due to the formation of large $\pi$-conjugation of polymer chains. After 2 h, a further increase of peak absorbance was observed, indicating the ongoing of enzymatic polymerization.

SEM images (Fig. 1B) show HRP–PTBA PBCs as aggregated nanoparticles, since the PBCs should form around enzyme molecules and the PBCs nanoparticles may aggregate somewhat during polymerization and especially after cast-coating. In addition, AuNPs and MWCNTs can also be encapsulated into the PBCs (Fig. 1C and D), since the nanomaterials can act as absorbants for enzyme (Cui et al., 2007; Zhang et al., 2004). The results indicate that enzymatic synthesis here is favorable for convenient preparation of conducting nanomaterials integrated PBCs.

The enzyme electrodes were prepared by cast-coating PBCs on Au electrodes, followed by coating with an outer-layer CS film, and the electrode modification was characterized by CV and EIS of Au electrodes, followed by coating with an outer-layer CS film, cules and the PBCs nanoparticles may aggregate somewhat during polymerization. Since the PBCs should form around enzyme molecules, as schematically shown in Scheme 1.

To obtain the best sensitivity for H$_2$O$_2$ assay, various conditions were optimized via variation of the examined one while others fixed, as shown in Fig. S3 with discussion details. The concentrations of HRP, TBA and Gox for PBCs' synthesis were optimized to be 2, 1.5 and 1 mg mL$^{-1}$, respectively. The concentration of K$_4$Fe(CN)$_6$, pH and applied potential for amperometric measurement were optimized to be 1 mM, 7.0 and $-0.1$ V, respectively. The amperometric responses of several HRP electrodes to H$_2$O$_2$ under optimum conditions are shown in Fig. 2B and Fig. S2. Excellent linear relationships are obtained in the concentration range from 1 to 300 μM ($r^2=0.997$) with sensitivity of 390 μA mM$^{-1}$ cm$^{-2}$, limit of detection (LOD) of 0.1 μM ($S/N=3$) and response time of 7 s for CS/HRP–PTBA/Au plate/Au. The CS/HRP–PTBA/Au plate/Au electrode exhibits a higher sensitivity than CS/HRP/PTBA/Au and CS/HRP–PTBA/Au, due to incorporation of polymer layer. After CS was cast-dried, the peak currents were increased obviously and the $\Delta E_p$ was decreased to 86 mV, due probably to the affinity between CS that is partially cationic at pH 7.0 and anionic Fe(CN)$_6^{3-/4-}$. The Nyquist plots are shown in Fig. S1B, the electron-transfer resistance ($R_{ct}$) for bare Au and Au plate/Au are estimated to be 216 and 95 Ω, respectively. Cast-coating the PBCs increased $R_{ct}$ to 1.98 kΩ, and the successive CS cast-coating decreased $R_{ct}$ to 293 Ω. The above EIS data validate the CV results, both indicating that the enzyme film has been successfully assembled on the Au electrode surface.

Due to the significance of H$_2$O$_2$ in biological systems and its practical applications, the development of efficient electrochemical H$_2$O$_2$ sensors holds a special attraction for researchers (Chen et al., 2012). K$_4$Fe(CN)$_6$ is adopted as mediator here due to its excellent electron transferability and a high degree of electrochemical reversibility (Li et al., 2008; Won et al., 2010). Fig. 2A shows first-cycle CV curves at CS/HRP–PTBA/Au plate/Au electrode in 0.1 M PBS containing 1 mM K$_4$Fe(CN)$_6$ and 0 or 0.2 mM H$_2$O$_2$. In the absence of H$_2$O$_2$, typical redox peaks of Fe(CN)$_6^{3-/4-}$ are observed. When 0.2 mM H$_2$O$_2$ was added, the CV curve exhibited enlarged cathodic current and lowered anodic current. At Au plate/Au electrode, CV curves changed negligibly after H$_2$O$_2$ addition, except for increased oxidation currents of H$_2$O$_2$ at high potentials. The enzyme electrode also displays a sharper and larger increase of open circuit potential after H$_2$O$_2$ addition than Au plate/Au (Fig. S2). The above results confirm a pronounced catalytic behavior of HRP, as schematically shown in Scheme 1.

Fig. 2. (A) CV curves (first cycle) at CS/HRP–PTBA/Au plate/Au and Au plate/Au (inset) electrodes in 0.1 M PBS containing 1 mM K$_4$Fe(CN)$_6$ and 0 (a) or 0.2 (b) mM H$_2$O$_2$. Scan rate: 50 mV s$^{-1}$. (B) Chronoamperometric response at $-0.10$ V and calibration curve (inset, with blowup from 1 to 50 μM) on CS/HRP–PTBA/Au plate/Au electrode to successive additions of H$_2$O$_2$ in 0.1 M PBS containing 1 mM K$_4$Fe(CN)$_6$. The linearly regressed line (blue) is also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
sensitivity of 451 μA mM⁻¹ cm⁻², owing to the improved electrochemical behavior of the conducting nanomaterials-integrated PBCs. Our biosensors give analytical performance among the best of recently reported amperometric H₂O₂ biosensors using mediators, as listed in Table 1.

The apparent Michaelis–Menten constant (K_app) is an indicator of the enzyme–substrate kinetics. According to the Lineweaver–Burk equation, K_app is obtained to be 0.13 mM at CS/HRP–PTBA/AuNPs/Au (Fig. S5, curve a), which is lower than those reported for other electrode configurations, such as HRP immobilized in AuNPs/self-doped polyaniline nanofibers (2.21 mM) (Chen et al., 2011a) or electropolymerized PTBA (1.22 mM) (Cui et al., 2011), indicating a suitable microenvironment here for enzyme immobilization. We evaluated the K_app of solution-state HRP by amperometric measurement. 0.1 M PBS containing 1 mM K₄Fe(CN)₆ and 100 μg mL⁻¹ H₂O₂ was used. H₂O₂ with different concentrations was added, and Ai in 60 s was recorded. As shown in Fig. S5 (curve b), according to the Lineweaver–Burk equation, the value of K_app for solution-state HRP was obtained to be 0.09 mM. The slight decrease of affinity for immobilized HRP (K_app=0.13 mM) may be due to the diffusion barrier of the substrates and slight conformational distortion of the encapsulated HRP.

The reproducibility, long-term stability and selectivity were also examined. The relative standard deviation (RSD) at CS/HRP–PTBA/AuNPs/Au was 4.7% for five successive measurements of 0.1 mM H₂O₂ using one enzyme electrode, illustrating good reproducibility. After being stored in PBS at 4 °C for a month, current signal decreased by less than 9.4% for CS/HRP–PTBA/AuNPs/Au, but decreased by 19.9% for CS/HRP/PTBA/AuNPs/Au, highlighting the better preservation of bioactivity in enzymatically synthesized polymer than chemically synthesized polymer (Fig. S6). The enhanced stability can be attributed to the fact that the construction process is mild, and no damage is inflicted on the enzyme molecules. As shown in Fig. S7, there were no obvious responses after additions of glucose, uric acid and ascorbic acid, displaying good anti-interference ability.

In addition, spectrophotometry was used to measure the activity of native and polymer-encapsulated HRP. Usually, one unit (U) of enzymatic activity of HRP is defined as consumption of 1 μmol H₂O₂ in 60 s (Cao et al., 2008), and ESA is defined as the ratio of the molar quantity of enzymatically consumed H₂O₂ in μmol in 60 s (nH₂O₂) to the mass of enzyme (mₑ) in mg. ESA is calculated as follows, ESA = nH₂O₂/mₑ = ΔA × V/[b × c × mₑ], where nH₂O₂ is obtained from enzymatically generated oxidized TMB that can be quantified by Lambert–Beer’s law; ΔA is the change of absorbance at 60 s; V is the volume of reaction solution/dispersion (here 0.008 L, see Section 2); b is the pathlength of absorbing medium (here 1 cm); c is the molar absorption coefficient of the product (5.9 × 10⁶ M⁻¹ cm⁻¹ for TMB at 450 nm (Joseph et al., 1982)); mₑ is the amount of HRP used in the reaction (here 0.3 × 10⁻³ mg obtained from solution dilution). The ΔA at 60 s was experimentally obtained as 0.642 ± 0.007 and 0.615 ± 0.009 for native HRP and HRP–PTBA PBCs, respectively. Hence, the ESA of native and encapsulated HRP are quantified as 290 ± 3 and 278 ± 4 U mg⁻¹, respectively, implying preservation of 95.8% bioactivity of native HRP in our PBCs. In addition, Fig. S8 shows the spectrophotometric kinetic curves of native and encapsulated HRP (note that the dispersion volume here was 0.003 L), and the small discrepancy witnesses their almost identical bioactivity. The excellent preservation of enzymatic activity in our PBCs is attributed to the mild encapsulation process and biocompatible microenvironment.

### Table 1

<table>
<thead>
<tr>
<th>Fabrication</th>
<th>Mediator</th>
<th>Sensitivity (μA mM⁻¹ cm⁻²)</th>
<th>LOD (μM)</th>
<th>Linear range (nM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP/PTBA–RTIL/GCE</td>
<td>HQ</td>
<td>~218.5</td>
<td>0.5</td>
<td>0.005 – 0.425</td>
<td>Cui et al. (2011)</td>
</tr>
<tr>
<td>HRP/HIL–GPN-TNT/GCE</td>
<td>HQ</td>
<td>230.0</td>
<td>2.2</td>
<td>0.015 – 0.75</td>
<td>Liu et al. (2012)</td>
</tr>
<tr>
<td>Au/SPAN–HRP–CS/GCE</td>
<td>HQ</td>
<td>289.6</td>
<td>1.6</td>
<td>0.01 – 2</td>
<td>Chen et al. (2011b)</td>
</tr>
<tr>
<td>HRP/polymers(GMA-co-VFc)/GCE</td>
<td>Fe</td>
<td>0.01</td>
<td>2.6</td>
<td>2 – 30</td>
<td>Şenel et al. (2010)</td>
</tr>
<tr>
<td>MB-SWNTH/HRP/GCE</td>
<td>MB</td>
<td>661.0</td>
<td>0.1</td>
<td>0.005 – 0.4</td>
<td>Palangsruntikul et al. (2010)</td>
</tr>
<tr>
<td>HRP/AuTiO₂/Ti</td>
<td>MB</td>
<td>~224.4</td>
<td>2</td>
<td>0.005 – 0.4</td>
<td>Kafi et al. (2008)</td>
</tr>
<tr>
<td>HRP/MDMS/GCE</td>
<td>HQ</td>
<td>403.6</td>
<td>0.078</td>
<td>0.0002 – 0.68</td>
<td>Zhang et al. (2007a)</td>
</tr>
<tr>
<td>HRP–SiO₂/Au</td>
<td>HQ</td>
<td>–</td>
<td>3</td>
<td>0.02 – 0.2</td>
<td>Yang et al. (2009)</td>
</tr>
<tr>
<td>HRP–ADA/µCDSH/Au</td>
<td>HQ</td>
<td>0.109</td>
<td>7</td>
<td>0.028 – 5.5</td>
<td>Camacho et al. (2009)</td>
</tr>
<tr>
<td>CSHMs/HRP–AuNPs–Fe(CN)₆⁻/–CHMs/Pt</td>
<td>K₄Fe(CN)₆</td>
<td>~107</td>
<td>0.8</td>
<td>0.0035 – 1.4</td>
<td>Li et al. (2008)</td>
</tr>
<tr>
<td>CS/HRP–PTBA/AuNPs/Au</td>
<td>K₄Fe(CN)₆</td>
<td>390</td>
<td>0.1</td>
<td>0.001 – 0.3</td>
<td>This work</td>
</tr>
<tr>
<td>CS/HRP–PTBA/AuNPs/Au</td>
<td>K₄Fe(CN)₆</td>
<td>376</td>
<td>0.1</td>
<td>0.001 – 0.5</td>
<td>This work</td>
</tr>
<tr>
<td>CS/HRP–PTBA/AuNPs/Au</td>
<td>K₄Fe(CN)₆</td>
<td>344</td>
<td>0.2</td>
<td>0.002 – 0.7</td>
<td>This work</td>
</tr>
<tr>
<td>CS/HRP–PTBA–AuNPs/AuNPs/Au</td>
<td>K₄Fe(CN)₆</td>
<td>402</td>
<td>0.1</td>
<td>0.001 – 0.65</td>
<td>This work</td>
</tr>
<tr>
<td>CS/HRP–PTBA–AuNPs/AuNPs/Au</td>
<td>K₄Fe(CN)₆</td>
<td>451</td>
<td>0.1</td>
<td>0.001 – 0.45</td>
<td>This work</td>
</tr>
</tbody>
</table>

GCE: glassy carbon electrode; HQ: hydroquinone; Fe: ferrocene; RTIL: room temperature ionic liquids; HIL: hydrophobic ionic liquid; TNT: TiO₂ nanotubes; Au/SPAN: gold nanoparticles/self-doped polyaniline nanofibers; MB: methylene blue; poly(GMA-co-VFc): poly(glycidylmethacrylate-co-vinylferrocene); MDMS: magnetic dextran microsphere; HRP–ADA: adamantane-modified HRP; pCDSH: polythiophenyl-½-cyclodextrin polymer and CSHMs: chitosan/silica sol-gel hybrid membranes.

3.2. Construction of bienzyme electrode for glucose detection

Our system can also be used to encapsulate other biomacromolecules for biosensing applications, and to our knowledge, this is the first report on self-encapsulated immobilization of oxidase plus peroxidase based on enzymatic polymerization. As shown in Scheme S1A, the bionzymatic PBCs triggered by H₂O₂ (Route I) or glucose (Route II) were synthesized. As shown in Scheme S1B, GOx catalyzes the oxidation of glucose in the presence of oxygen to form H₂O₂, which immediately oxidizes Fe(CN)₆⁻ to Fe(CN)₃⁻ in the presence of HRP, and the electroreduction of Fe(CN)₃⁻ is used for monitoring the overall reaction. Fig. 3A shows the first-cycle CV responses at CS/GOx–HRP–PTBA(H₂O₂)/Auplate/Au to glucose in 0.1 M PBS containing 1.0 mM K₄Fe(CN)₆. When 0.5 mM glucose was added, the CV curve exhibited somewhat enlarged cathodic current and lowered anodic current while very minor changes occur at the Auplate/Au electrode after addition of glucose, displaying a pronounced catalytic behavior of the oxidase-peroxidase reaction. Likewise, the CS/GOx–HRP–PTBA(H₂O₂)/Auplate/Au electrode displayed a larger change of open circuit potential after addition of glucose than the Auplate/Au electrode (Fig. S9).

It should be noted that Fe(CN)₃⁻ can hardly mediate the turnover of GOx in our experiments, as reported previously (Calvo et al., 1996; Chen et al., 2011a). Since the Fe(CN)₃⁻ mediator failed to directly turn over GOx in our experiments...
The present system favorably avoid the “unusual amperometric responses” observed when other “bifunctional” mediators that can also efficiently turn over GOx (e.g., ferrocene (Matsumoto et al., 2002), toluidine blue O (Wang et al., 2010), benzoquinone (BQ), are used, as shown in Fig. S10 with discussion details. We thus believe that the choice of monofunctional mediator Fe(CN)$_6^{3-}$ has suggested a new way to avoid quantitative complicity during bienzyme-coupled biosensing applications. Steady-state current responses at CS/GOx–HRP–PTBA(H$_2$O$_2$)/Au(plate)/Au and CS/GOx–HRP–PTBA(glucose)/Au(plate)/Au are shown in Fig. 3B. The two biosensors exhibit sensitivity values of 75.1 and 61.5 μA·mmol$^{-1}$·cm$^{-2}$, respectively, and the enzyme polymerization protocol using added H$_2$O$_2$ is somewhat better. It may be attributed to the fact that the generation of H$_2$O$_2$ by GOx (Route II) is limited by low-concentration dissolved oxygen available, which slows down the rate of enzymatic polymerization. The performance of CS/GOx–HRP–PTBA(H$_2$O$_2$)/Au(plate)/Au is among the best of previous reports (Table S1). Furthermore, the bienzyme biosensor displays good enzyme-substrate affinity with lower value of K$_{MP}$ as 1.87 mM, as well as good anti-interference ability (Fig. S11). The reproducibility at CS/GOx–HRP–PTBA(H$_2$O$_2$)/Au(plate)/Au was also checked by five successive measurements, with an acceptable RSD of 4.3%. There was no significant decrease in response in several days and only an 11.6% loss of original response was observed after a month of storage, implying that the enzymes were stably immobilized with its bioactivity well preserved.

4. Conclusions

In summary, we have suggested a facile enzymatic polymerization protocol to prepare enzyme-PTBA PBCs for high-performance mono-/bi-enzyme amperometric biosensing. Enzyme immobilization based on boronic acid-diols interaction is beneficial for bioactivity preservation, as confirmed by UV–vis spectrophotometric tests. The thus-prepared mono-/bi-enzyme biosensors exhibit high sensitivity, low LOD, and good storage stability. Nanomaterials can also be entrapped conveniently into the PBCs to improve analytical performance to some degree. The present enzymepolymerization protocol may be conveniently extended to other biosensing and bioreactor systems. The successful choice of monofunctional mediator Fe(CN)$_6^{3-}$ here may be of some reference value to guide biosensor design of other oxidase-peroxidase systems for sensitive and simple (not unusual) biosensing readout.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (21175042, 21075036, 21105026, 20975038), Hunan Lotus Scholars Program, the Foundations of Hunan Provincial Education Department, Program for Science and Technology Innovative Research Team in Higher Educational Institutions of Hunan Province, State Key Laboratories of Chemo/Biosensing and Chemometrics (200902).

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.2013.01.001.

References


