

# Zirconia Nanoparticles Enhanced Grafted Collagen Tri-Helix Scaffold for Unmediated Biosensing of Hydrogen Peroxide

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A novel, biocompatible, thermally steady, and nontoxic zirconia enhanced grafted collagen tri-helix scaffold was prepared on a graphite electrode. This scaffold provided a microenvironment for loading biomolecules and helped to retain their natural structure. UV-vis spectroscopy and scanning electron microscopy were used to characterize the scaffold and the structure of immobilized biomolecules. Using horseradish peroxidase (HRP) as an example, this scaffold accelerated its electron transfer and led to its direct electrochemical behavior with a good thermal stability up to 80 °C. The surface electron-transfer rate constant of the immobilized HRP was  $(5.55 \pm 0.43) \text{ s}^{-1}$  in 0.1 M pH 7.0 PBS at 18 °C. The immobilized HRP showed an electrocatalytic activity to the reduction of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) without aid of an electron mediator. The linear response range of the biosensor for  $\text{H}_2\text{O}_2$  was from 1.0 to 73.0  $\mu\text{M}$  with a correlation coefficient of 0.999 ( $n = 14$ ), a limit of detection down to 0.25  $\mu\text{M}$  and an apparent Michaelis-Menten constant of  $(0.28 \pm 0.02) \text{ mM}$ . The biosensor exhibited high sensitivity, acceptable stability, and reproducibility. The  $\text{ZrO}_2$  grafted collagen provided an excellent matrix for protein immobilization and biosensor preparation.

## Introduction

Recently, the use of nanosized materials for the design of biosensors has received considerable attraction. The immobilization of proteins on nanostructured materials such as colloidal gold,<sup>1</sup> montmorillonite,<sup>2</sup> clay,<sup>3,4</sup> mesoporous materials,<sup>5</sup> and molecular sieves<sup>6</sup> has been identified as a very promising method for biosensing applications. Some oxide nanoparticles such as  $\text{SiO}_2$ ,<sup>7</sup>  $\text{ZrO}_2$ ,<sup>8</sup> and  $\text{MnO}_2$ <sup>9</sup> can also be used for immobilizing proteins and accelerating the electron transfer between the immobilized proteins and the electrodes. Through the layer-by-layer assembly, Hu et al.<sup>7</sup> immobilized some kinds of heme proteins on  $\text{SiO}_2$  nanoparticles and investigated the driving forces for the assembly procedure. These studies show that the nanosized materials possess good biocompatibility, high active surface areas for protein loading, regular structures, and good mechanical, thermal, and chemical stability. After being immobilized on these matrices, the redox proteins show enhanced electrochemical activity, which allows the electrochemical measurements of their substrates with high sensitivity and improved selectivity.<sup>10,11</sup>

The combination of enzyme modified nanoparticles with some functional materials such as redox polymers can be used for the

design of reagentless amperometric biosensors. A redox polymer-carbon nanotube-enzyme composite has been prepared for the preparation of glucose biosensors.<sup>12</sup> The development of new advanced hybrid materials, particularly nanoparticle-biocompatible inorganic porous materials, leads to another biosensing application based on the direct electron transfer of immobilized redox proteins. A porous gold nanoparticle-calcium carbonate hybrid material has been fabricated for the assembly of horseradish peroxidase (HRP), which is immobilized on a glassy carbon electrode with silica sol-gel.<sup>13</sup> However, the presence of silica sol-gel hinders the electron transfer between the immobilized HRP and electrode surface, though this process can be accelerated by the gold nanoparticles coexisting on the electrode surface. Thus the cyclic voltammogram corresponding to the direct electrochemical behavior shows relatively large difference of peak potentials and the formed biosensor shows low sensitivity. To overcome this limitation it is necessary to develop a new method to fabricate nanoparticles enhanced hybrid materials for biosensing application.

This work proposed a novel hybrid material prepared with zirconia and grafted collagen for direct immobilization and electron transfer of redox proteins. Collagen is one of the biopolymers most extensively used to construct functionalized hybrid structures. Its stalks consist of right-handed supercoils of three left-handed polyproline II-type helices with major sequences of (Gly-Pro-Hyp) $_n$ .<sup>14</sup> To strengthen its mechanical and thermal stability, extensive efforts have been made to mimic<sup>15</sup> or stabilize<sup>16</sup> its soft conformation. Owing to the abundant -OH and -NH<sub>2</sub>

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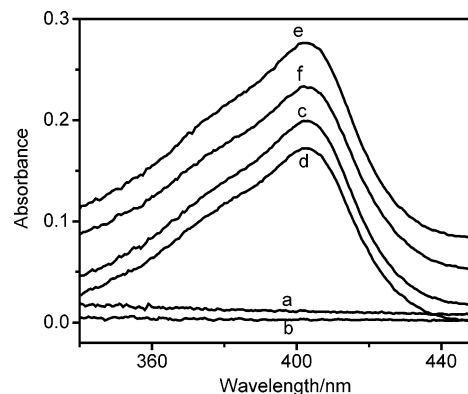
groups,<sup>17</sup> the collagen molecule shows good affinity to metal oxide for stabilizing metal oxide nanoparticles away from their aggregation.<sup>14</sup> By grafting the collagen molecule with some lipophilic materials such as methyl methacrylate (MMA), the noticeable improvement in biocompatibility of collagen has been obtained.<sup>18</sup> The hybrid material prepared in this work showed good biocompatibility and thermal and mechanical stability, which could conveniently form a layer of membranes on the electrode surface for the immobilization of redox proteins. The presence of nanosized zirconia enhanced the tri-helix scaffold of collagen, increased the loading of biomolecules, and accelerated the electron transfer of the immobilized redox proteins. Using HRP as a model redox protein, the as-synthesized zirconia-grafted collagen hybrid material was used for studying the direct electrochemistry of redox proteins and the preparation of relatively sensitive biosensors. The unique three-dimensional porous structure prevented the leaking of enzymes and led to a good preparation reproducibility of the sensor. The prepared biosensor for hydrogen peroxide showed good analytical performance, indicating that the metal oxide nanoparticle-grafted collagen hybrid materials were a sort of biomaterial suitable for protein immobilization and preparation of the third generation biosensors.

### Experimental Procedures

**Chemicals and Solvents.** Horseradish peroxidase (EC 1.11.1.7, >250 U mg<sup>-1</sup>) and collagen were purchased from Shanghai Biotechnology Co. Ltd. (China) and Taozheng Bioengineering Technology Co. Ltd. (Beijing, China), respectively, and used without further purification. Zirconium *n*-propoxide was purchased from Fluka. Other reagents were of analytical reagent grade. The 0.1 M phosphate buffer solutions with different pH values were prepared by mixing the stock standard solutions of Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub> and adjusting the pH with 0.1 M H<sub>3</sub>PO<sub>4</sub> or NaOH. All solutions were prepared with twice-distilled water.

ZrO<sub>2</sub> and grafted collagen were prepared according to refs 19 and 20, respectively. The zirconia enhanced grafted collagen tri-helix scaffold was prepared by dispersing ZrO<sub>2</sub> powder and grafting collagen in alcohol, which was stirred overnight and refluxed at 60 °C for 8 h. The mixture was refrigerated for 2 h at -18 °C to remove impurities and then separated with the vacuumized filtration in room temperature to obtain the enhanced tri-helix scaffold (ZrO<sub>2</sub>-grafted collagen powder).

**Construction of HRP-Zirconia Enhanced Tri-Helix Scaffold.** The homemade graphite electrodes (GE, 6.0 mm in diameter) were first polished with a 1.0, 0.3, and 0.05 μm α-alumina slurry (Beuhler), respectively, rinsed thoroughly with doubly distilled water between each polishing step, and then sonicated in 1:1 nitric acid, acetone, and doubly distilled water successively and allowed to dry at room temperature. The ZrO<sub>2</sub>-grafted collagen suspension was obtained by dispersing 4.0 mg of ZrO<sub>2</sub>-grafted collagen powder in 1.0 mL of dimethyl sulfoxide (DMSO). After mixing 10 μL of the HRP solution (5 mg mL<sup>-1</sup>) with 5.0 μL of the ZrO<sub>2</sub>-grafted collagen suspension, the mixture was cast on the graphite electrode. With the slow evaporation of solvent and aging step overnight in a sealed flask at a constant temperature of 18 °C, the HRP-zirconia enhanced tri-helix scaffold was obtained on a graphite electrode (HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE). Alternatively, only 10 μL of the HRP solution, ZrO<sub>2</sub>-grafted collagen suspension, or HRP/DMSO solution with the same HRP concentration was cast onto the electrodes to form HRP/GE, ZrO<sub>2</sub>-grafted collagen/DMSO/GE or HRP/DMSO/GE, respectively. Prior to electrochemical experiments, the modified



**Figure 1.** UV-vis spectra of DMSO (a), ZrO<sub>2</sub>-grafted collagen/DMSO (b), HRP (c), HRP/DMSO (d), HRP/ZrO<sub>2</sub>-grafted collagen (e), and HRP/ZrO<sub>2</sub>-grafted collagen/DMSO (f) solutions.

electrodes were rinsed thoroughly with doubly distilled water and kept in 0.1 M pH 7.0 PBS at 4 °C.

**Apparatus and Procedures.** Electrochemical measurements were performed on a CHI 730A electrochemical analyzer (CHI Co., China) at (18 ± 2) °C with a conventional three-electrode system using the modified electrode as a working electrode, a platinum wire as an auxiliary electrode, and a saturated calomel electrode (SCE) as reference against which all potentials were measured. The thermal stability of the HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE was examined by increasing the cell temperature and keeping the corresponding temperature for 20 min and then recording the cyclic voltammograms. The amperometric measurements were performed in a stirred cell at (18 ± 2) °C by applying a potential of -350 mV with successive additions of H<sub>2</sub>O<sub>2</sub> solution into the buffer solution. The sensor response was measured as the difference between total and residual currents. All experimental solutions were deoxygenated by bubbling highly pure nitrogen for 15 min and maintained under nitrogen atmosphere during measurements.

UV-vis absorbance spectroscopy was performed with a UV-vis-3100-Nir Recording Spectrophotometer (Shimadzu, Japan). The morphologies of the enzyme modified electrodes were characterized using scanning electron microscopy (SEM, LEO 1530 VP, Germany) at an acceleration voltage of 5 kV. A gold film was applied by argon plasma sputtering for 30 s to the specimens for SEM measurements.

### Results and Discussion

**Characterization of HRP Immobilized in ZrO<sub>2</sub>-Grafted Collagen/DMSO Membranes.** Figure 1 shows the UV-vis spectra of different solutions. The spectra in the presence of HRP display the maximum absorption around 403 nm (curves d, f, e, and c in Figure 1), while no absorption is observed in the absence of HRP (curves a and b in Figure 1). Obviously, this absorption peak is attributed to the Soret band of HRP, which would diminish upon the full protein denaturation.<sup>21</sup> The small shift in the absorption peak indicates an interaction between ZrO<sub>2</sub>-grafted collagen and HRP molecules due to the surface potential energy and absorption properties of ZrO<sub>2</sub>-grafted collagen. Such an interaction does not destroy the structure and change the fundamental microenvironment of HRP.

The response of an enzyme electrode is related to its physical morphology. Thus, the surface morphology of the ZrO<sub>2</sub>-grafted collagen matrix is an important factor affecting its performance. Figure 2 shows the SEM images of different membranes. The micrograph of ZrO<sub>2</sub>-grafted collagen/DMSO displayed a chemically clean sponge-like unique three-dimensional porous structure. This three-dimensional structure showed a very narrow particle size distribution with the average diameter ranging from 30 to 40 nm (Figure 2a). In the absence of both DMSO and the three-

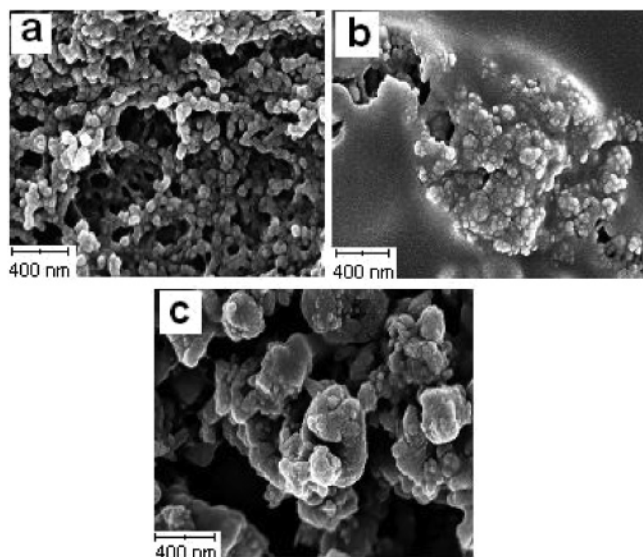
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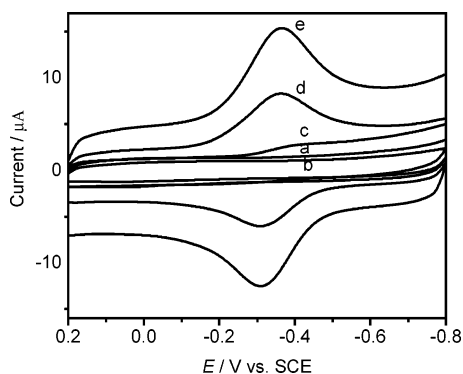
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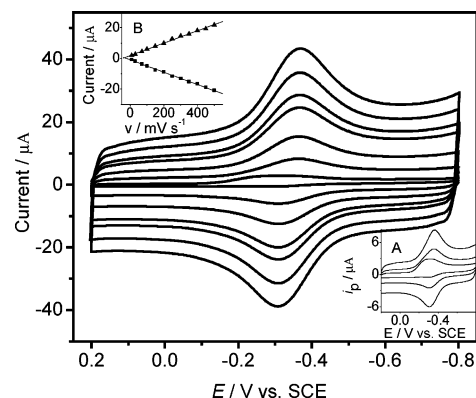
**Figure 2.** Scanning electron micrographs of ZrO<sub>2</sub>-grafted collagen/DMSO (a), HRP (b), and HRP/ZrO<sub>2</sub>-grafted collagen/DMSO (c) films on a glass slice.



**Figure 3.** Cyclic voltammograms of GE (a), ZrO<sub>2</sub>-grafted collagen/DMSO/GE (b), HRP/GE (c), HRP/DMSO/GE (d), and HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE (e) in 0.1 M pH 7.0 PBS at 100 mV s<sup>-1</sup>.

dimensional porous structure, the HRP molecules aggregated together (Figure 2b). However, a well-distributed layer of the HRP could be formed by mixing it with ZrO<sub>2</sub>-grafted collagen/DMSO (Figure 2c). The nanoparticles in the ZrO<sub>2</sub>-grafted collagen composite were surrounded by HRP molecules, leading to bigger size of nanoparticles. The scanning electron microscopic images proved that the ZrO<sub>2</sub>-grafted collagen provided an excellent matrix for protein immobilization. The enhanced tri-helix scaffold of hybrid material was chemically clean and porous with a very narrow size distribution of the nanoparticles, which was due to the abundant -OH and -NH<sub>2</sub> groups of the collagen molecule<sup>17</sup> to stabilize the metal oxide nanoparticles away from their aggregation.<sup>14</sup> The grafting of collagen improved the biocompatibility of the hybrid material.<sup>18</sup> The presence of metal oxide nanoparticles increased the homogeneous loading of enzyme molecules, provided a good preparation reproducibility of the immobilized HRP electrodes, and prevented the leaking of enzyme. Thus, the prepared biosensors showed good preparation reproducibility and stability.

**Direct Electrochemistry of HRP Immobilized in ZrO<sub>2</sub>-Grafted Collagen/DMSO Films.** Figure 3 shows the cyclic voltammograms of different electrodes in 0.1 M pH 7.0 PBS at 100 mV s<sup>-1</sup>. No obvious electrochemical response was observed at both GE and ZrO<sub>2</sub>-grafted collagen/DMSO/GE (curves a and b in Figure 3). The presence of the ZrO<sub>2</sub>-grafted collagen/DMSO membrane resulted in a low background current. The HRP/ZrO<sub>2</sub>-

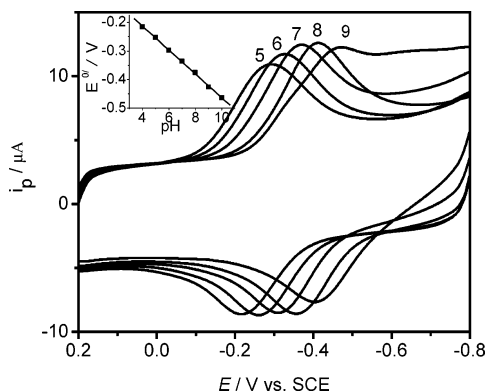


**Figure 4.** Cyclic voltammograms of HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE in 0.1 M pH 7.0 PBS at 10, 70, 150, 250, 300, 400, and 500 mV s<sup>-1</sup> (from lowest to highest peak current). Insets: cyclic voltammograms of this system at 10, 30, and 70 mV s<sup>-1</sup> (A) and plot of peak current vs scan rate (B).

grafted collagen/DMSO/GE gave a couple of stable and well-defined redox peaks at -309 and -365 mV (curve e in Figure 3), while the HRP/GE displayed only a reduction peak (curve c in Figure 3). Obviously, these peaks were attributed to the reduction and oxidation of the electroactive center of the immobilized HRP. Although the HRP/DMSO/GE also displayed a couple of redox peaks of HRP (curve d in Figure 3), these peak currents were smaller than those of the HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE. The appearance of these redox peaks in the presence of DMSO was attributed to the decrease of the dielectric constant of the microenvironment around HRP molecules, which decreased the reorganization energy of biological electron transfer.<sup>22</sup> ZrO<sub>2</sub> nanoparticles in the hybrid material not only enhanced the tri-helix scaffold but also provided a microenvironment for preserving the natural structure and accelerating the electron transfer of the immobilized redox proteins. In the novel coating, the immobilized HRP showed a greatly improved direct electrochemical behavior (Figure 3). The formal potential of the heme Fe<sup>III/II</sup> couple in HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE, estimated as the midpoint of reduction and oxidation potentials, was  $-337 \pm 3$  mV in 0.1 M pH 7.0 PBS. This value was similar to those of -0.33<sup>23</sup> and -0.345 V,<sup>24</sup> suggesting that most molecules preserved their native structure after being entrapped in the ZrO<sub>2</sub>-grafted collagen. The formal potential was more negative than that of -0.22 V obtained in an aqueous solution,<sup>25</sup> indicating that the presence of the ZrO<sub>2</sub>-grafted collagen stabilized the oxidized form of HRP.

With the increasing scan rate from 10 to 500 mV s<sup>-1</sup>, the reduction and oxidation peak currents of the HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE with a ratio of about 1:1 increased linearly, and the peak potentials showed a small shift (Figure 4), indicating a surface-controlled electrode process. The peak-to-peak separation of the cyclic voltammogram in 0.1 M pH 7.0 PBS at 100 mV s<sup>-1</sup> was 56 mV, which was smaller than that of 88 mV for HRP immobilized in the gold nanoparticle-CaCO<sub>3</sub> hybrid material.<sup>13</sup> From the peak-to-peak separations of the cyclic voltammograms at scan rates ranging from 70 to 500 mV s<sup>-1</sup> (Figure 4), the electron-transfer rate constant  $k_s$  of the HRP immobilized in the zirconia enhanced tri-helix scaffold was estimated according to the model of Laviron<sup>26</sup> with the formula

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**Figure 5.** Cyclic voltammograms of HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE in PBS with various pH values at 100 mV s<sup>-1</sup>. Inset: plot of formal potential vs pH.

$k_s = mnFv/RT$  to be  $(5.55 \pm 0.43) \text{ s}^{-1}$ , where  $m$  is a parameter related to the peak-to-peak separation,  $F$  is Faraday's constant,  $R$  is the gas constant,  $T$  is the temperature, and  $n$  is the number of electron transfers. Here,  $T = 291 \text{ K}$  and  $n = 1$ . This value was larger than those of  $1.13 \text{ s}^{-1}$  for HRP immobilized in DNA film,<sup>27</sup>  $0.92 \text{ s}^{-1}$  for HRP immobilized on the hexagonal mesoporous silica matrix,<sup>5</sup> and  $0.66 \text{ s}^{-1}$  for HRP coated on a sealing film-covered graphite electrode,<sup>28</sup> suggesting a reasonably fast electron transfer between the immobilized HRP and the electrode due to the presence of ZrO<sub>2</sub>-grafted collagen and DMSO.

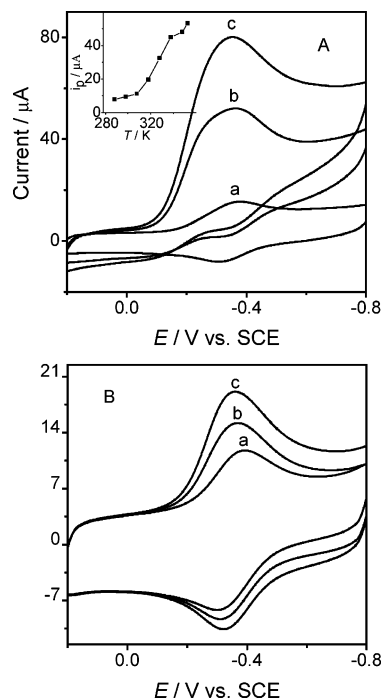
From the integration of the reduction peaks of the HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE at different scan rates, an average surface coverage of HRP was calculated to be  $(3.14 \pm 0.02) \times 10^{-10} \text{ mol cm}^{-2}$ , which was much larger than those of  $3.05 \times 10^{-11} \text{ mol cm}^{-2}$  entrapped in an agarose hydrogel film,<sup>24</sup>  $1.68 \times 10^{-11} \text{ mol cm}^{-2}$  entrapped in the methyl cellulose film,<sup>29</sup>  $(2.51 \pm 0.45) \times 10^{-11} \text{ mol cm}^{-2}$  immobilized on active carbon,<sup>30</sup>  $1.2 \times 10^{-12} \text{ mol cm}^{-2}$  immobilized in a conducting polymer,<sup>31</sup> and  $5 \times 10^{-11} \text{ mol cm}^{-2}$  entrapped in a biomembrane-like surfactant film,<sup>32</sup> indicating a high loading of enzyme molecules.

#### Effect of Solution pH on Direct Electron Transfer of HRP.

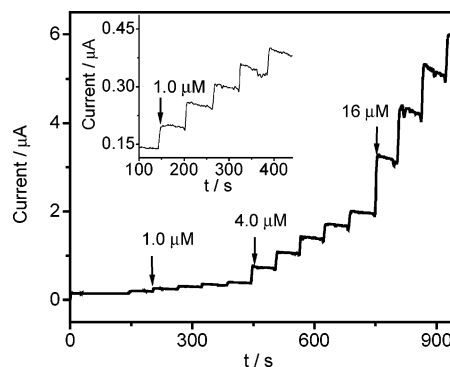
Figure 5 shows the effect of solution pH on the response of HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE. With the increase of the solution pH from 4.0 to 10.0, the negative shifts of both reduction and oxidation peak potentials were observed. In general, all changes in the peak potentials and currents with solution pH were reversible in the pH range. The plot of the formal potential (the average of the anodic and cathodic peak potentials) versus pH showed a slope of  $-41.8 \text{ mV pH}^{-1}$  with a correlation coefficient of 0.9994 (inset in Figure 5). The slope was close to the expected value of  $-57.8 \text{ mV pH}^{-1}$  at 291 K, indicating that one proton participated in the electron-transfer process for neutralizing the charge change during redox reaction.<sup>33</sup> Furthermore, the change of solution pH did not affect the peak-to-peak separation; thus, the diffusion of proton in the enhanced tri-helix scaffold of hybrid material was very fast.

#### Thermal Stability of HRP/ZrO<sub>2</sub>-Grafted Collagen/DMSO/GE.

Figure 6A shows the cyclic voltammograms of the HRP/



**Figure 6.** Cyclic voltammograms of HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE in 0.1 M pH 7.0 PBS at 15 (a), 55 (b), and 80 °C (c) in panel A and 0.1 M pH 7.0 PBS containing 0 (a), 70 (b), and 130 μM (c) H<sub>2</sub>O<sub>2</sub> at 18 °C in panel B at 100 mV s<sup>-1</sup>. Inset in panel A: plot of cathodic peak current vs temperature.



**Figure 7.** Amperometric response of the sensor at  $-350 \text{ mV}$  upon successive additions of 1.0, 4.0, and  $16 \mu\text{M}$  H<sub>2</sub>O<sub>2</sub> to pH 7.0 PBS. Inset: magnification for additions of  $1.0 \mu\text{M}$  H<sub>2</sub>O<sub>2</sub>.

ZrO<sub>2</sub>-grafted collagen/DMSO/GE in 0.1 M pH 7.0 PBS at various temperatures. With the increasing temperature from 15 to 80 °C, the cathodic peak current of the immobilized HRP increased, while the cathodic peak currents of the HRP/GE began to decrease when the temperature was up to 50 °C. The HRP in the ZrO<sub>2</sub>-grafted collagen/DMSO showed better thermal stability than on the bare graphite electrode. If the temperature was over 80 °C, some milk-white globules were observed on the electrode surface, the immobilized HRP lost its bioactivity. It was evident that the immobilized HRP had good thermal stability because of the unchanging microenvironment. These results indicated that this biosensor could handle a wide range of temperatures. The increase in the thermal stability of the HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE came from the good biocompatibility of grafted collagen and the presence of ZrO<sub>2</sub> nanoparticles.<sup>8</sup>

**Electrocatalysis of HRP/ZrO<sub>2</sub>-Grafted Collagen/DMSO/GE to Reduction of H<sub>2</sub>O<sub>2</sub>.** Upon the addition of H<sub>2</sub>O<sub>2</sub> to 0.1 M pH 7.0 PBS, the cyclic voltammogram of the immobilized HRP changed dramatically with an increase of reduction current

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**Table 1. Interference of External Matters to Response of the HRP/ZrO<sub>2</sub>-Grafted Collagen/DMSO/GE to 50  $\mu$ M Hydrogen Peroxide in 0.1 M pH 7.0 PBS**

external matters	concentration spiked ( $\mu$ M)	response change (%)	relative standard deviation ( $n = 6$ ) (%)
SO <sub>4</sub> <sup>2-</sup>	500	-1.3	0.8
CO <sub>3</sub> <sup>2-</sup>	500	4.3	1.1
ClO <sub>3</sub> <sup>-</sup>	500	8.4	1.8
Cl <sup>-</sup>	500	9.0	1.5
Br <sup>-</sup>	500	6.9	1.8
Fe <sup>3+</sup>	250	13.0	2.5
glycin	500	6.8	1.7
ascorbic acid	500	-0.4	1.2

and a decrease of oxidation current (Figure 6B), displaying obvious electrocatalytic behavior of the HRP to the reduction of H<sub>2</sub>O<sub>2</sub>.

The amperometric response of the sensor to H<sub>2</sub>O<sub>2</sub> at an applied potential of -350 mV upon successive additions of H<sub>2</sub>O<sub>2</sub> into stirring 0.1 M pH 7.0 PBS is shown in Figure 7. Upon the addition of an aliquot of H<sub>2</sub>O<sub>2</sub> to the buffer solution, the reduction current increased steeply to reach a stable value. The modified electrode achieved 95% of the maximum steady-state current in less than 5 s. The results demonstrated clearly that the electrocatalytic response was very fast. Although the current step for the catalyzed signal displayed a decreasing current over time, we did not observe the difference among the signals determined for several times at the same concentration of H<sub>2</sub>O<sub>2</sub>. The calibration curve of the sensor to H<sub>2</sub>O<sub>2</sub> concentration showed a linear range from 1.0 to 73.0  $\mu$ M. The linear regression equation was  $i$  ( $\mu$ A) = -0.0179 + 0.07372*c* ( $\mu$ M) with a correlation coefficient of 0.999 ( $n = 14$ ). On the basis of the fast direct electron transfer and electrocatalytic behavior of the immobilized HRP to the reduction of H<sub>2</sub>O<sub>2</sub>, the HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE showed a sensitivity of 0.26 A M<sup>-1</sup> cm<sup>-2</sup> and a limit of detection of 0.25  $\mu$ M for biosensing of H<sub>2</sub>O<sub>2</sub>, showing a high sensitivity. The sensitivity was much higher than that of 0.0017 A M<sup>-1</sup> cm<sup>-2</sup> for HRP in the porous gold nanoparticle-CaCO<sub>3</sub> hybrid material immobilized with silica sol-gel.<sup>13</sup>

When the concentration of H<sub>2</sub>O<sub>2</sub> was higher than 73  $\mu$ M, the steady-state amperometric response showed a characteristic of the Michaelis-Menten kinetic mechanism. The apparent Michaelis-Menten constant ( $K_M^{app}$ ), a reflection of both the enzymatic affinity and the ratio of microscopic kinetic constants, was obtained from the electrochemical version of the Lineweaver-Burk equation<sup>34</sup> to be (0.28  $\pm$  0.02) mM. This value was smaller than those of 1.38 mM for HRP immobilized in poly(ethylene glycol),<sup>35</sup> 2.3 mM for HRP immobilized on a colloid/cysteamine modified gold electrode,<sup>36</sup> and 5.5 mM for HRP immobilized in polymer.<sup>37</sup> Thus, the presence of ZrO<sub>2</sub>-grafted collagen and the enhanced tri-helix scaffold improved the affinity of immobilized HRP to H<sub>2</sub>O<sub>2</sub>.

**Real Sample Analysis.** With the purpose to verify the applicability of the proposed biosensor for real sample analysis,

35 and 55  $\mu$ M hydrogen peroxide was added into rainwater samples, respectively. The average recovery of the biosensor was 107% ( $n = 5$ ) and 99.1% ( $n = 5$ ), respectively. The rainwater sample without adding H<sub>2</sub>O<sub>2</sub> did not show any detectable signal.

The possible interference of foreign matters, which might occur in real samples, was tested. The results were shown in Table 1. When the concentrations of SO<sub>4</sub><sup>2-</sup>, CO<sub>3</sub><sup>2-</sup>, ClO<sub>3</sub><sup>-</sup>, Cl<sup>-</sup>, Br<sup>-</sup>, glycin, and ascorbic acid coexisting in the sample were 10 times that of hydrogen peroxide, no significant interference could be observed, indicating that these species did not affect the determination of hydrogen peroxide. However, Fe<sup>3+</sup> might be a main interference to HRP for the electrocatalytic reduction of hydrogen peroxide. When the concentration of Fe<sup>3+</sup> was 5 times that of hydrogen peroxide, the peak current showed an increase of approximately 13%.

**Stability and Reproducibility of the H<sub>2</sub>O<sub>2</sub> Sensor.** The direct electrochemistry of the HRP/ZrO<sub>2</sub>-grafted collagen/DMSO/GE could retain the constant current values with the continuous cyclic sweep in 0.1 M pH 7.0 PBS in the potential range from -0.8 to +0.2 V at 100 mV s<sup>-1</sup>. After 40 measurements, the immobilized HRP lost only 8.0% of its initial activity. When the sensor was not in use, it was stored in 0.1 M pH 7.0 PBS at 4 °C. This sensor was also more stable than those reported previously.<sup>8,13</sup> It retained 95% of its initial current response after a storage period of 2 months, while the hemoglobin on zirconia nanoparticles could retain 91% of the initial response,<sup>8</sup> which benefited from the good biocompatibility of the grafted collagen. The relative standard deviation was 1.8% for six successive determinations at a H<sub>2</sub>O<sub>2</sub> concentration of 60  $\mu$ M. The fabrication of five electrodes, made independently, showed a good reproducibility with a relative standard deviation of 2.7% for the currents determined at a H<sub>2</sub>O<sub>2</sub> concentration of 60  $\mu$ M. Thus, the ZrO<sub>2</sub>-grafted collagen was very efficient for retaining the electrocatalytic activity of HRP and preventing it from leaking out of the biosensor.

## Conclusions

ZrO<sub>2</sub>-grafted collagen is a good biocompatible hybrid material for the immobilization of redox proteins. Its porous tri-helix scaffold containing nanoparticles is effective for the high loading of biomolecules and tunneling electrons between the immobilized protein and electrode with a good thermal stability and a high affinity to enzyme substrates. This matrix can retain the bioactivity of the immobilized proteins to a large extent. The biosensor based on the direct electron transfer of the immobilized HRP shows good analytical performance, including high sensitivity, good precision, and acceptable fabrication reproducibility and storage stability. ZrO<sub>2</sub>-grafted collagen provides a promising application of hybrid materials for the study of direct electron transfer of proteins and the development of biosensors.

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