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 ± 0.43) × 10^10 M^(-1) 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 (H_2O_2) without aid of an electron mediator. The linear response range of the biosensor for H_2O_2 was from 1.0 to 73.0 μM with a correlation coefficient of 0.999 (n = 14), a limit of detection down to 0.25 μM and an apparent Michaelis–Menten constant of (0.28 ± 0.02) mM. The biosensor exhibited high sensitivity, acceptable stability, and reproducibility.

The 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 attention. The immobilization of proteins on nanostructured materials such as colloidal gold, montmorillonite, clay, mesoporous materials, and molecular sieves has been identified as a very promising method for biosensing applications. Some oxide nanoparticles such as SiO_2, ZrO_2, and MnO_2 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. immobilized some kinds of heme proteins on 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.

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. The development of new advanced hybrid materials, particularly nanoparticle–bio-compatible 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. 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). To strengthen its mechanical and thermal stability, extensive efforts have been made to mimic or stabilize its soft conformation. Owing to the abundant --OH and --NH_2

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groups, the collagen molecule shows good affinity to metal oxide for stabilizing metal oxide nanoparticles away from their aggregation. By grafting the collagen molecule with some lipophilic materials such as methyl methacrylate (MMA), the noticeable improvement in biocompatibility of collagen has been obtained. 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 relox 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 relox proteins. Using HRP as a model relox protein, the as-synthesized zirconia-grafted collagen hybrid material was used for studying the direct electrochemistry of relox 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−1) and collagen were purchased from Shanghai Biotechnology Co. Ltd. (China) and Taizheng 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 Na2HPO4 and NaH2PO4 and adjusting the pH with 0.1 M H3PO4 or NaOH. All solutions were prepared with twice-distilled water.

ZrO2 and grafted collagen were prepared according to refs 19 and 20, respectively. The zirconia enhanced grafted collagen tri-helix scaffold was prepared by dispersing ZrO2 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 (ZrO2-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 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 ZrO2-grafted collagen suspension was obtained by dispersing 4.0 mg of ZrO2-grafted collagen powder in 1.0 mL of dimethyl sulfoxide (DMSO). After mixing 10 µL of the HRP solution (5 mg mL−1) with 5.0 µL of the ZrO2-grafted collagen suspension, the mixture was cast on the graphite electrode. With the slow evaporation of solvent and aging step overnight in a sealed atmosphere during measurements.

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. The small shift in the absorption peak indicates an interaction between ZrO2-grafted collagen and HRP molecules due to the surface potential energy and absorption properties of ZrO2-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 ZrO2-grafted collagen matrix is an important factor affecting its performance. Figure 2 shows the SEM images of different membranes. The micrograph of ZrO2-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-
at both GE and ZrO$_2$-grafted collagen/DMSO/GE (curves a and b in Figure 3). The grafting of collagen improved the biocompatibility of the hybrid material. The presence of metal oxide nanoparticles, which was due to the abundant $-OH$ and $-NH_2$ groups of the collagen molecule$^{17}$, to stabilize the metal oxide nanoparticles away from their aggregation. The grafting of collagen improved the biocompatibility of the hybrid material. 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$_2$-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$^{-1}$.

With the increasing scan rate from 10 to 500 mV s$^{-1}$, the reduction and oxidation peak currents of the HRP/ZrO$_2$-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$^{-1}$ was 56 mV, which was smaller than that of 88 mV for the HRP/DMSO/GE in an aqueous solution, suggesting that most molecules preserved their native structure after being entrapped in the ZrO$_2$-grafted collagen. The peak-to-peak separation of the cyclic voltammogram in 0.1 M pH 7.0 PBS was at 100 mV s$^{-1}$.

**Figure 2.** Scanning electron micrographs of ZrO$_2$-grafted collagen/DMSO (a), HRP (b), and HRP/ZrO$_2$-grafted collagen/DMSO (c) films on a glass slice.

**Figure 3.** Cyclic voltammograms of GE (a), ZrO$_2$-grafted collagen/DMSO/GE (b), HRP/GE (c), HRP/DMSO/GE (d), and HRP/ZrO$_2$-grafted collagen/DMSO/GE (e) in 0.1 M pH 7.0 PBS at 100 mV s$^{-1}$.

**Figure 4.** Cyclic voltammograms of HRP/ZrO$_2$-grafted collagen/DMSO/GE in 0.1 M pH 7.0 PBS at 10, 70, 150, 250, 300, 400, and 500 mV s$^{-1}$ (from lowest to highest peak current). Insets: cyclic voltammograms of this system at 10, 30, and 70 mV s$^{-1}$ (A) and plot of peak current vs scan rate (B).

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\[ k_s = mnFv/RT \]

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, \( T = 291 \ degrees \ K \) and \( n = 1 \). This value was larger than those of 1.13 \( s^{-1} \) for HRP immobilized in DNA film,\(^{27} \) 0.92 \( s^{-1} \) for HRP immobilized on the hexagonal mesoporous silica matrix,\(^{5} \) and 0.66 \( s^{-1} \) for HRP coated on a sealing film-covered graphite electrode,\(^{28} \) suggesting a reasonably fast electron transfer between the immobilized HRP and the electrode due to the presence of ZrO\(_2\)–grafted collagen and DMSO.

From the integration of the reduction peaks of the HRP/ZrO\(_2\)-grafted collagen/DMSO/GE at different scan rates, an average surface coverage of HRP was calculated to be \((3.14 \pm 0.02) \times 10^{-10} \ mol \ cm^{-2}\), which was much larger than those of \(3.05 \times 10^{-11} \ mol \ cm^{-2}\) entrapped in an agarose hydrogel film,\(^{24} \) \(1.68 \times 10^{-11} \ mol \ cm^{-2}\) immobilized in the methyl cellulose film,\(^{29} \) \((2.51 \pm 0.45) \times 10^{-11} \ mol \ cm^{-2}\) immobilized on active carbon,\(^{26} \) \(1.2 \times 10^{-12} \ mol \ cm^{-2}\) immobilized in a conducting polymer,\(^{31} \) and \(5 \times 10^{-11} \ mol \ cm^{-2}\) entrapped in a biomembrane-like surfactant film,\(^{32} \) 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\(_2\)-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 \(-41.8 \ mV \ pH^{-1}\) with a correlation coefficient of 0.9994 (inset in Figure 5). The slope was close to the expected value of \(-57.8 \ mV \ pH^{-1}\) at 291 K, indicating that one proton participated in the electron-transfer process for neutralizing the charge change during redox reaction.\(^{33} \) 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\(_2\)-Grafted Collagen/DMSO/GE.**

Figure 6A shows the cyclic voltammograms of the HRP/ZrO\(_2\)-grafted collagen/DMSO/GE in PBS with various pH values at 100 mV s\(^{-1}\). Inset: plot of formal potential vs pH.

\[ k_s = mnFv/RT \]

\( T \) is the gas constant, \( R \) is the gas constant, \( T \) is the temperature, and \( n \) is the number of electron transfers. Here, \( T = 291 \ degrees \ K \) and \( n = 1 \). This value was larger than those of 1.13 \( s^{-1} \) for HRP immobilized in DNA film,\(^{27} \) 0.92 \( s^{-1} \) for HRP immobilized on the hexagonal mesoporous silica matrix,\(^{5} \) and 0.66 \( s^{-1} \) for HRP coated on a sealing film-covered graphite electrode,\(^{28} \) suggesting a reasonably fast electron transfer between the immobilized HRP and the electrode due to the presence of ZrO\(_2\)–grafted collagen and DMSO.

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**Thermal Stability of HRP/ZrO\(_2\)-Grafted Collagen/DMSO/GE.**

Figure 6A shows the cyclic voltammograms of the HRP/
of H2 O2, the HRP/ZrO2-grafted collagen/DMSO/GE showed a
electrocatalytic behavior of the immobilized HRP to the reduction
14). On the basis of the fast direct electron transfer and
glycol),35 2.3 mM for HRP immobilized on a colloid/cysteamine
than those of 1.38 mM for HRP immobilized in poly(ethylene
sensitivity was much higher than that of 0.0017 A M-
immobilized with silica sol
the Michaelis
M app ), a reflection of both the enzymatic
M) a, M
-0.02) mM. This value was smaller
stirring 0.1 M pH 7.0 PBS is shown in Figure 7. Upon the addition
of an aliquot of H2 O2 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 H2O2. The calibration curve of
the sensor to H2O2 concentration showed a linear range from 1.0 to
73.0 M. The linear regression equation was i (mA) = −0.0179
+ 0.07372c (mM) 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 H2O2, the HRP/ZrO2-grafted collagen/DMSO/GE showed a
sensitivity of 0.26 A M−1 cm−2 and a limit of detection of 0.25
µM for biosensing of H2O2, showing a high sensitivity. The
sensitivity was much higher than that of 0.0017 A M−1 cm−2 for
HRP in the porous gold nanoparticle–CaCO3 hybrid material
immobilized with silica sol−gel.13
When the concentration of H2O2 was higher than 73 µM, the
steady-state amperometric response showed a characteristic of
the Michaelis–Menten kinetic mechanism. The apparent Michaelis–
Menten constant (Km)99, a reflection of both the enzymatic
affinity and the ratio of microscopic kinetic constants, was
obtained from the electrochemical version of the Lineweaver–
Burk equation14 to be (0.28 ± 0.02) mM. This value was smaller
than those of 1.38 mM for HRP immobilized in poly(ethylene
glycol),35 2.3 mM for HRP immobilized on a colloid/cysteamine
modified gold electrode,36 and 5.5 mM for HRP immobilized in
polymer.37 Thus, the presence of ZrO2-grafted collagen and the
enhanced tri-helix scaffold improved the affinity of immobilized
HRP to H2O2.
Real Sample Analysis. With the purpose to verify the
applicability of the proposed biosensor for real sample analysis,
Table 1. Interference of External Matters to Response of the
HRP/ZrO2-Grafted Collagen/DMSO/GE to 50 µM Hydrogen
Peroxide in 0.1 M pH 7.0 PBS

<table>
<thead>
<tr>
<th>external matters</th>
<th>concentration spiked (µM)</th>
<th>response change (%)</th>
<th>relative standard deviation (n = 6) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO2−</td>
<td>500</td>
<td>−1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>CO2−</td>
<td>500</td>
<td>4.3</td>
<td>1.1</td>
</tr>
<tr>
<td>ClO−</td>
<td>500</td>
<td>8.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Cl−</td>
<td>500</td>
<td>9.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Br−</td>
<td>500</td>
<td>6.9</td>
<td>1.8</td>
</tr>
<tr>
<td>Fe3+</td>
<td>250</td>
<td>13.0</td>
<td>2.5</td>
</tr>
<tr>
<td>glycin</td>
<td>500</td>
<td>6.8</td>
<td>1.7</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>500</td>
<td>−0.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

and a decrease of oxidation current (Figure 6B), displaying
obvious electrocatalytic behavior of the HRP to the reduction of
H2O2.

The amperometric response of the sensor to H2O2 at an applied
potential of −350 mV upon successive additions of H2O2 into
stirring 0.1 M pH 7.0 PBS is shown in Figure 7. Upon the addition
of an aliquot of H2O2 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
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HRP to H2O2.

Conclusions
ZrO2-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. ZrO2-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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