Hollow TiO₂ modified reduced graphene oxide microspheres encapsulating

hemoglobin for a mediator-free biosensor

Hui Liu^{*}, Kai Guo, Congyue Duan, Xiaonan Dong, Jiaojiao Gao

School of Materials Science and Engineering, Shaanxi University of Science and
Technology, Xi'an 710021, P. R. China

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^{*}Corresponding author. Tel: +86-29-86177018; Fax: +86-29-86177018.

Email address: liuhui@sust.edu.cn (Hui Liu)

Abstract

Hollow TiO₂ modified reduced graphene oxide microspheres (hollow TiO₂-rGO microspheres or H-TiO₂-rGO MS) have been synthesized and then be used to immobilize hemoglobin (Hb) to fabricate a mediator-free biosensor. The morphology and structure of hollow TiO₂-rGO microspheres were characterized by scanning electron microscopy, transmission electronic microscopy and X-ray diffraction. Results of spectroscopy and electrochemistry tests revealed that hollow TiO₂-rGO microsphere is an excellent immobilization matrix with biocompatibility for redox protein, affording good protein bioactivity and stability. The hollow TiO₂-rGO microspheres with special structure and component enhance the immobilization efficiency of proteins and facilitate the direct electron transfer, which result in the better H₂O₂ detection performance-the wide linear range of 0.1-360 μ M for H₂O₂ (sensitivity of 417.6 μ A mM⁻¹ cm⁻²) and the extremely low detection limit of 10 nM for H₂O₂. Moreover, the hollow microsphere can provide a protective microenvironment for Hb to make the as-prepared biosensor improve long-term stability. The as-prepared biosensor retains 95.4% of the initial response to H₂O₂ after 60-day storage. Hence, this work suggests that if can be fabricated a mediator-free biosensor, hollow TiO₂-rGO microspheres will find wide potential applications in environmental analysis and biomedical detection.

Keywords: Graphene, Hollow microsphere, Direct electrochemistry, Biosensor, Long-term stability

1. Introduction

Graphene, an atomic sheet of sp^2 bonded carbon atoms, possesses a high specific surface area ($\sim 2600 \text{ m}^2\text{g}^{-1}$), extraordinary electronic transport properties ($20,000 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$) and high electrocatalytic activities (Akhavan and Ghaderi 2009; Allen Mattew et al. 2010; Park et al. 2008; Park and Ruoff 2009). Furthermore, graphene with the unique two-dimensional crystal structure can anchor or wrap metal and metal oxide nanoparticles effectively to form many kinds of nanocomposites, offering versatile selective catalytic or sensing performances (Dong et al. 2010; Lee et al. 2012; Liu et al. 2012; Shan et al. 2010; Zhang et al. 2010). TiO_2 -graphene nanocomposites have drawn great attention among many kinds of nanocomposites because TiO_2 owns superior photocatalytic performance, good biocompatibility, pollution-free environment and chemical and thermal stabilities (Fan et al. 2011; Liu et al. 2013; Liu and Chen 2005; Sun et al. 2013; Wang et al. 2015). Recent studies demonstrated that TiO_2 -graphene nanocomposite is a promising electrode material for electrochemical sensing applications (Cao et al. 2013; Jang et al. 2012; Liu et al. 2014a). In these biosensors, TiO_2 -graphene, facilitating the direct electron transfer and enhancing the catalytic activity of enzymes, was always employed as a matrix for immobilizing enzymes. Meanwhile, Kim and coworkers reported that the immobilization quality of enzyme, which determines the property of biosensor, is influenced by the morphology and structure of nanocomposites (Kim et al. 2006; Xie et al. 2011). Although substantial effort has been devoted to the development of new nanostructured materials for use in biosensors, achieving new morphologies of TiO_2 -graphene nanocomposites with high performance is still a challenge (Xie et al. 2011).

In this paper, the hollow TiO_2 modified reduced graphene oxide microspheres (hollow TiO_2 -rGO microspheres or H- TiO_2 -rGO MS) were synthesized successfully. The bag-like structure make hollow microsphere with a breach as a matrix of enzymes has many advantages as follows. Firstly, as shown in

1 Fig. 1, the inner space of the “bag” allows more quantity of enzymes to be immobilized. Meanwhile,
2 some enzyme can be absorbed on outside of the hollow rGO sphere. Secondly, TiO_2 nanoparticles with
3 excellent biocompatibility can provide a protective microenvironment for the enzymes to retain their
4 enzymatic stability and activity and the nanoscaled TiO_2 particles (less than 10 nm in diameter) can
5 greatly enhance the active surface area which is available for adsorbing protein. Thirdly, the substrate
6 can easily be entrapped by graphene with a high surface area. Some of the absorbed substrate has the
7 chance to pass the defects on rGO to get into the hollow sphere resulting in substrate's enrichment. The
8 rGO is converted from GO by hydrothermal method and rGO's two dimensional crystal structure has
9 lots of defects. Concentration of substrate and enzyme in this confined region will increase the chance
10 of effective collisions between them (Xie et al. 2011). Finally, graphene, for its excellent mobility of
11 charge carriers, is able to become an excellent media to promote electrical communication between
12 enzyme and electrode. Thus, hollow TiO_2 -rGO microsphere is a promising support for enzyme
13 immobilization and has potential applications in biosensors.

14 In this work, hemoglobin (Hb) was selected as a model redox protein to construct a biosensor. The
15 performance of a sensor composed of Hb immobilized in the hollow TiO_2 -rGO microspheres was
16 compared with that of sensors based on TiO_2 , rGO, solid TiO_2 @rGO microspheres and sheet TiO_2 -rGO
17 nanocomposite in order to highlight the advantages of the hollow TiO_2 -rGO microsphere as a support.

18 **2. Experimental**

19 **2.1. Materials and apparatus**

20 Hb (from bovine blood) and Nafion solution (5 wt% in lower aliphatic alcohols) are purchased from
21 Sigma. Unless otherwise noted, all other chemicals (analytical grade) are purchased from Sinopharm
22 Chemical Reagent Co., Ltd., China. In all experiments, deionized water is used.

1 Field-emission scanning electron microscopy (FE-SEM) is performed using a Hitachi S-4800 &
2 Hiroba energy dispersive X-ray electron microscopy. Transmission electronic microscopy (TEM)
3 images are obtained on a Hitachi H-800 instrument. X-ray diffraction (XRD) patterns are recorded on a
4 Rigaku D/max 2200pc diffractometer using Cu K α radiation of wavelength $\lambda = 0.15418$ nm at 40 kV
5 and 40 mA. Raman spectra are taken using an ALPHA 300M Micro-Raman spectrometer system.
6 UV-vis spectra are recorded using a PerkinElmer Lambda-950 spectrophotometer. All electrochemical
7 experiments are performed with a CHI 660D electrochemical workstation (CH Instruments, Shanghai,
8 China). A conventional three-electrode system which consists of a platinum wire as the counter
9 electrode, an Ag/AgCl/3M KCl as the reference electrode and a 3-mm diameter modified glassy carbon
10 electrode (GCE) as the working electrode is used in all electrochemical experiments. Unless otherwise
11 noted, 0.1 M phosphate buffered saline (PBS; pH 7.0) is used as the supporting electrolyte in all
12 experiments. The buffer is purged with highly purified nitrogen for at least 30 min and a nitrogen
13 atmosphere environment is kept in electrochemical measurements.

14 **2.2 Preparation of hollow TiO₂-rGO microspheres**

15 Graphene oxide (GO) was prepared from graphite powder by the modified Hummers method
16 (Hummers Jr and Offeman 1958; Kovtyukhova et al. 1999), while TiO₂ precursor microspheres and
17 TiO₂@GO microspheres were prepared by the method in our previous references (Liu et al. 2013). The
18 typical preparation steps of hollow TiO₂-rGO microspheres are shown in Fig. 2A. The preparation of
19 hollow TiO₂-rGO microspheres adopts HCl etching reaction under the hydrothermal condition. In this
20 preparation, the 1 g TiO₂@GO microspheres were dispersed in 70mL mixture of ethanol and HCl
21 solution ($V_{[\text{ethanol}]}: V_{[\text{HCl}]} = 1:1$) by high-speed stirring. After a high-speed stirring about 15 minutes, the
22 suspension was loaded in a 100 mL Teflon-lined autoclave. The autoclave was sealed and then heated

in an oven of 180 °C for 24 h. As a result, black flocculent products (hollow TiO₂-rGO microspheres) were formed. Next, the flocculent products were collected, washed with ethanol and distilled water for several times to remove ions and possible remnants and dried at 50 °C. For comparison, solid TiO₂@rGO microspheres (TiO₂@rGO MS) were synthesized by the similar procedure without HCl solution (Fig. S1). Meanwhile, sheet TiO₂-rGO nanocomposite was prepared by the method in our previous references (Fig. S2) (Liu et al. 2014b).

2.3. Preparation of enzyme electrode

To investigate the electrochemical behavior of H-TiO₂-rGO MS based biosensor, enzyme electrode is assembled. Prior to use, the GCE was sequentially polished using 1.0, 0.3 and 0.05 µm alumina powder on a polishing cloth and rinsing with deionised water. The electrode is then sonicated in ethanol and deionised water. Next, the electrode is dried using a purified nitrogen stream. The enzyme electrode was prepared by a simple casting method. Firstly, 1 mL of 2 mg mL⁻¹ H-TiO₂-rGO MS suspension and 0.5 mL of 10 mg mL⁻¹ Hb PBS are mixed and then stirred for 20 min. Next, 0.5 mL of Nafion (5%) was added to the mixture and then stirred for 10 min. A suspension (denoted as Suspension I) finally contains a concentration of 2.5 mg mL⁻¹ Hb, 1 mg mL⁻¹ H-TiO₂-rGO MS and 1.25% Nafion. Finally, 4 µL of Suspension I is dropped to the surface of a freshly polished GCE to prepare the Nafion/Hb/H-TiO₂-rGOMS/GCE. A beaker is covered over the electrode so that water can evaporate slowly in air and a uniform film electrode can be obtained. The dried Nafion/Hb/H-TiO₂-rGOMS/ GCE are stored at 4°C in refrigerator when the electrode is not use.

For comparison, TiO₂, rGO, solid TiO₂@rGO MS and sheet TiO₂-rGO are used for the fabrication of Nafion/Hb/TiO₂/GCE, Nafion/Hb/rGO/GCE, Nafion/Hb/TiO₂@rGOMS/GCE and Nafion/Hb/TiO₂-rGO/GCE through similar procedures as described above. In the control experiment,

1 Nafion/Hb/GCE and Nafion/H-TiO₂-rGOMS/GCE are also prepared. Before electrochemical
2 measurements, all the as-prepared film electrodes are immersed in pH 7.0 PBS for 30 min to remove
3 residual components.

4 **3. Results and discussions**

5 **3.1. Characterization of hollow TiO₂-rGO microspheres**

6 Fig. 2 displays schematic illustration of synthesis steps of hollow TiO₂-rGO microspheres and
7 corresponding typical SEM or TEM images of the samples prepared at various stages. As shown in Fig.
8 2A, the synthesis steps are mainly as follows: firstly, TiO₂ microspheres were synthesized by a
9 controlled hydrolysis of tetrabutyl titanate in ethanol. Secondly, the TiO₂ microspheres were directly
10 wrapped by graphene oxide (GO) nanosheets to form TiO₂@GO (Liu et al. 2013). Finally, the hollow
11 TiO₂-rGO microspheres were synthesized by HCl etching reaction under the hydrothermal condition.
12 From Fig. 2B and C, it is observed that TiO₂ microspheres show relatively uniform morphology with
13 diameters about 1 μm and the TiO₂ microspheres own smooth surface (Fig. 2C). Fig. 2D and E presents
14 that the spherical morphology of TiO₂@GO microspheres were not changed after the wrapped process.
15 It's worth noting that different from that of the pure TiO₂ microspheres, the surface of the TiO₂@GO
16 microspheres presents a wrinkle-like morphology, which indicates that the surface of TiO₂ microsphere
17 was wrapped by GO nanosheets (Fig. 2E). From Fig. 2F and G, the hollow microspheres have been
18 formed after hydrothermal treatment in HCl solution resulting from its etching reaction to TiO₂.
19 However, graphene sheets remain lots of TiO₂ nanoparticles because etching reaction cannot fully
20 remove TiO₂. Interestingly, almost every hollow microsphere has a breach, which can act as a channel
21 for material to migrate outside, in other word, the hollow microsphere with a breach can form a
22 bag-like structure, which is very benefit for enzyme's encapsulation. Meanwhile, nanoscaled TiO₂

1 particles not only have plentiful active surface area available to adsorb enzyme, but also have good
2 biocompatibility to offer protective microenvironment for enzyme to keep its bioactivity. Hence, the
3 hollow TiO₂-rGO microsphere is an excellent matrix for immobilizing enzyme.

4 Fig. S3 presents XRD patterns of GO (curve a), rGO (curve b), H-TiO₂-rGO MS (curve c) and TiO₂
5 (curve d) respectively. It can be seen that the XRD pattern of GO (curve a) shows a major peak at $2\theta =$
6 10.5° , corresponding to a d-spacing of 0.841 nm, which is much larger than the d-spacing of natural
7 graphite at 0.335 nm. This change indicates that graphite is oxidized to form GO (Sher Shah et al.
8 2012). After hydrothermal treatment, this peak disappears and a new peak appears centered at $2\theta =$
9 24.2° , corresponding to the 0.367 nm d-spacing of graphene (curve b), indicating the reduction of GO
10 to rGO (Bai et al. 2009). Curve c in Fig. S3 exhibits the XRD pattern of the as-prepared H-TiO₂-rGO
11 MS, all the diffraction peaks are in good agreement with that of anatase TiO₂ (curve d). However, the
12 diffraction peaks of rGO belong to H-TiO₂-rGO MS are not distinguishable in XRD patterns. This
13 phenomenon can be ascribed to the much lower crystalline extent of rGO than that of TiO₂, which leads
14 to the shielding of the rGO peaks by those of TiO₂ (Fan et al. 2011; Sher Shah et al. 2012; Zhang et al.
15 2009).

16 Fig. S4 presents Raman spectra of GO (curve a), rGO (curve b), TiO₂ (curve c) and H-TiO₂-rGO MS
17 (curve d) respectively. All of GO, rGO and H-TiO₂-rGO MS show the same Raman shift at 1355 and
18 1598 cm^{-1} corresponding to the D- and G-bands of graphene respectively. However, it can be found
19 that the D/G intensity ratio increases from 0.99 (GO) to 1.10 (H-TiO₂-rGO MS or rGO) after the
20 hydrothermal treatment, which can be attributed to the increase in the number of smaller sp² domains
21 and a re-establishment of conjugated graphene networks (rearomatization) (Stankovich et al. 2007),
22 suggesting a successful transformation of GO to rGO (Kudin et al. 2008; Xiang et al. 2012).

Meanwhile, the increase of D-bond intensity demonstrates the increase of graphene's defects (Pham et al. 2011). These defects, the accesses of substrate to enter the inner hollow sphere, are benefit for enzymatic reaction. The Raman spectra of TiO_2 (curve c) shows the presence of peaks at 158, 408, 513 and 630 cm^{-1} can be attributed to the $E_{g(1)}$, $B_{1g(1)}$, $A_{1g}+B_{1g(2)}$, and $E_{g(2)}$ modes of anatase phase of TiO_2 (Xiang et al. 2012; Yu et al. 2011). Curve d show all the Raman bands for anatase phase of TiO_2 and rGO, confirms the co-existence of anatase TiO_2 and rGO in the nanocomposite.

3.2. Encapsulation of Hb in the Nafion/Hb/H- TiO_2 -rGO MS composite film

Fig. S5A exhibits a typical TEM image of Hb/H- TiO_2 -rGO MS. Due to the high surface area and the bag-like structure of the hollow microsphere, H- TiO_2 -rGO MS loads a lot of Hb. Fig. S5B depicts the microstructure image of Nafion/Hb/H- TiO_2 -rGOMS composite film. It is noted that the hollow microsphere with a bag-like structure is discernible on the surface of modified electrode. H- TiO_2 -rGO MS was embedded in the Nafion to form a stable composite film, which was essential for the stability of the prepared enzyme electrode. Moreover, because of the high surface area of H- TiO_2 -rGO MS, the effective surface area of the modified electrode improves greatly.

The stability of Hb immobilized both in H- TiO_2 -rGO MS and Nafion/Hb/H- TiO_2 -rGOMS composite film was investigated by UV-vis spectroscopy (Sun et al. 2014a; Wang et al. 2015). The Soret absorption bands of heme proteins provide information about the conformational integrity of the proteins. As shown in Fig. S5C, the Soret absorption band of Hb immobilized in H- TiO_2 -rGO MS (curve a) and Nafion/Hb/H- TiO_2 -rGOMS composite film (curve b) is located at about 405 nm which is close to that of native Hb at 406 nm (curve c), indicating the Hb retained the essential features of its conformational integrity, also revealing that hollow TiO_2 -rGO microspheres have biocompatibility and did not suffer significant protein denaturation.

3.3. Electrochemical characteristics of the modified electrodes

Fig. 3 shows the typical cyclic voltammograms (CVs) of different modified electrodes in 0.1 M PBS (pH 7.0) at a scan rate of 0.1 V s^{-1} . No redox peak is observed at Nafion/H-TiO₂-rGOMS/GCE (curve a), which indicates that Nafion/H-TiO₂-rGOMS/GCE is electroinactive in the given potential window. The Nafion/Hb/H-TiO₂-rGOMS/GCE (curve g) exhibits a couple of stable and well-defined redox peaks at -0.369 V and -0.334 V vs. Ag/AgCl, which can be ascribed to direct electron transfer (DET) between Hb and the underlying electrode (Sun et al. 2013). The formal potential ($E^0 = (E_{p,a} + E_{p,c})/2$) of Hb is -0.351 V vs. Ag/AgCl, in agreement with -0.345 V and -0.353 V vs. Ag/AgCl reported for the Hb(Fe^{III/II}) redox couple in Hb-ZnO-MWCNTs-Nafion (Ma and Tian 2010) and Hb-TiO₂-MXene-Nafion (Wang et al. 2015) films. Compared with that of the Nafion/Hb/H-TiO₂-rGOMS/GCE, the redox peaks observed at the Nafion/Hb/GCE (curve c, in Fig. 3) are 87.3% smaller, suggesting an enhanced electron transfer between Hb molecules and the surface of the Nafion/Hb/H-TiO₂-rGOMS/GCE. Meanwhile, the much smaller redox peaks of Nafion/Hb/GCE can be attributed to the denaturation of proteins on the bare electrode surface and the difficulty for proteins realizing the DET process on the bare electrode (Dong et al. 2011a; Wang et al. 2015; Zhu et al. 2011). It can be clearly seen from Fig. 3 that direct electron transfer between Hb molecules and GCE is greatly enhanced at the Nafion/Hb/H-TiO₂-rGOMS/GCE. For comparison, Nafion/Hb/TiO₂/GCE (curve c), Nafion/Hb/rGO/GCE (curve d), Nafion/Hb/TiO₂-rGO/GCE (curve e) and Nafion/Hb/TiO₂@rGO MS/GCE (curve f) have also been carried out. The redox peaks of these four electrodes are smaller than that of Nafion/Hb/H-TiO₂-rGOMS/GCE. It can conclude that hollow TiO₂-rGO microspheres have better effect on the facilitation of electron transfer, which may resulted from their unique morphology and structure.

Fig. S6 shows the cyclic voltammograms of Nafion/Hb/H-TiO₂-rGOMS/GCE at 0.1 M PBS (pH 7.0) with the scan rate increasing from 0.1 to 0.8 V s⁻¹. With the increase of the scan rate, the cathodic and anodic peak currents of Hb increase simultaneously and the cathodic and anodic peak potentials show a small shift resulting in the peak to peak separation slightly enlarged. The inset of Fig. S6 shows that the cathodic (i_{pc}) and anodic (i_{pa}) peak currents increase linearly with scan rates increased from 0.1 to 0.8 V s⁻¹. It reveals that the electrode reaction corresponds to a surface attached reaction process. Moreover, the slopes of anodic and cathodic processes are nearly equal, suggesting that Hb is stable and not undergoing denaturation. Using the Laviron method for a surface-controlled electrochemical system (Laviron 1979), the apparent heterogeneous electron transfer rate constant (k_s) of Hb immobilized on Nafion/Hb/H-TiO₂-rGOMS/GCE is estimated about 3.27 s⁻¹, which is higher than the value reported for Hb immobilized TiO₂ nanorods-graphene (0.65 s⁻¹) (Sun et al. 2013) and nitrogen-doped graphene (2.36s⁻¹) (Sun et al. 2014b), suggesting a faster electron-transfer process. This means that hollow TiO₂-rGO microspheres facilitates the direct electron transfer of Hb.

The average surface concentration of electroactive Hb (Γ^* , mol cm⁻²) can be estimated from the charge integration of the reduction peak of the CV using the formula:

$$Q = nF\Gamma^* \quad (1)$$

where F is Faraday constant, Q is the charge (C) and can be obtained by integrating the reduction peak of Hb, n and A stand for the number of electron transferred and the geometrical surface area of the electrode, respectively. According to this method, the surface concentration of electroactive Hb (Γ^*) at Nafion/Hb/H-TiO₂-rGOMS/GCE was calculated as 5.26×10⁻¹⁰ mol cm⁻², which is much greater than the theoretical value for monolayer coverage (1.89×10⁻¹¹ mol cm⁻²) (Wang et al. 2005). This means that multilayer of Hb entrapped in the Nafion/Hb/H-TiO₂-rGOMS composite film participates in the

electron-transfer process. Meanwhile, the surface concentration of electroactive Hb of Nafion/Hb/H-TiO₂-rGOMS/GCE is higher than that of Nafion/Hb/TiO₂@rGOMS/GCE (4.30×10^{-10} mol cm⁻²) and Nafion/Hb/TiO₂-rGO/GCE (3.82×10^{-10} mol cm⁻²), revealing that hollow TiO₂-rGO microspheres can immobilize more Hb and retain its activity and stability on the surface of GCE.

Fig. S7 shows that the cyclic voltammograms of Nafion/Hb/H-TiO₂-rGOMS/GCE in different pH values 0.1 M PBS. Stable and well-defined CVs are observed in the pH values range 6.0~8.0. With the increase of pH value, both the cathodic and anodic peaks shifted to the negative. This can be contributed to the involvement of proton transfer in the Hb(Fe^{III})/Hb(Fe^{II}) redox couple (Chen et al. 2015). The $E^{o'}$ value of Hb varied linearly in the range of pH values of 6.0~8.0, with a slop of -54.2 mV pH⁻¹ (the inset of Fig. S7). This value is very close to the theoretical value for the transfer of one proton and electron in a reversible reduction (-58 mV pH⁻¹ at 25 °C) (Chen et al. 2015; Xie et al. 2011).

3.4. Electrocatalytic properties of the Nafion/Hb/H-TiO₂-rGOMS/GCE

Hb usually exhibits electrocatalytic activity for H₂O₂, nitrite and Cl₃CCOOH after Hb with retained bioactivity is immobilized on an electrode surface (Dong et al. 2011a; Lu et al. 2008; Sun et al. 2013). Using H₂O₂ as a probe, we investigated the electrocatalytic properties of the Nafion/Hb/H-TiO₂-rGOMS/GCE. As shown in Fig. 4, the reduction peak increases dramatically with the increasing of H₂O₂ concentration and the oxidation peak decreases and disappears display the obvious electrocatalytic behavior of Hb for the reduction of H₂O₂. A possible reaction mechanism of H₂O₂ catalyzed by the Hb-based electrode is postulated as follows (George and Lee 2009):

With the increasing of H₂O₂ concentration, the current value tents to saturation limit (the inset of

Fig. 4) and the saturation behavior is characteristic of enzyme-based catalysis. The apparent Michaelis-Menten constant (K_M^{app}) can be obtained by the electrochemical-version of Lineweaver-Burk equation (Kamin and Wilson 1980):

$$\frac{1}{I_{ss}} = \frac{K_M^{app}}{I_{max}C} + \frac{1}{I_{max}} \quad (4)$$

where I_{ss} is the steady-state current after the addition of substrate, C is the bulk concentration of the substrate, and I_{max} is the maximum current measured under saturated substrate conditions. The K_M^{app} value of Nafion/Hb/H-TiO₂-rGOMS/GCE is estimated to be 141.4 μ M (curve a in Fig. S8). This value is smaller than those ever reported values of 180 μ M (Dong et al. 2011b) and 379 μ M (Sun et al. 2012), indicating that Hb immobilized on Nafion/Hb/H-TiO₂-rGOMS/GCE retained higher bioactivity and had a high biological affinity to H₂O₂. Note that the K_M^{app} value of Nafion/Hb/H-TiO₂-rGOMS/GCE is also smaller than that of Nafion/Hb/TiO₂@rGOMS/GC (184.9 μ M) and Nafion/Hb/TiO₂-rGO/GCE (198.2 μ M) (curve b and c in Fig. S8, respectively), which reveals that this hollow structure is more favorable to make immobilized Hb keep bioactive.

3.5. Biosensor performance of the Nafion/Hb/H-TiO₂-rGOMS/GCE

The biosensor performance for the detection of H₂O₂ of the Nafion/Hb/H-TiO₂-rGOMS/GCE was also investigated through amperometry method. Fig. 5 gives the amperometric responses of the modified glassy carbon electrodes at -0.35 V as adding H₂O₂ in stirred PBS (pH 7.0). Fig. 5A demonstrates that, on Nafion/Hb/H-TiO₂-rGOMS/GCE, the response time (achieving 95% of the steady-state current) is less than 3 s. And Fig. 5B indicates that, for Nafion/Hb/H-TiO₂-rGOMS/GCE, a well-defined linear relationship between the current and the H₂O₂ concentration is observed. The calibration plot shows a linear range from 0.1 to 360 μ M (correlation coefficient of 0.999, N=24) with a sensitivity of 417.6 μ A mM⁻¹ cm⁻² and a detection limit of 10 nM (based on a signal-to-noise ratio of 3).

Performances of various H_2O_2 biosensors are listed in Table S1. It is clear that the performance of Nafion/Hb/H-TiO₂-rGOMS/GCE is significantly improved. Furthermore, as can be seen from Fig. S9 and Table S2, compared with Nafion/Hb/TiO₂-rGO/GCE and Nafion/Hb/TiO₂@rGOMS/GCE, the H-TiO₂-rGO MS based biosensor has better performance. There are some reasons why the Nafion/Hb/H-TiO₂-rGOMS/GCE displays both a lower detection limit and a wider linear range. Firstly, the microsphere's hollow structure allows a lot of enzymes to be immobilized. Secondly, TiO₂ nanoparticles with excellent biocompatibility can provide a protective microenvironment for the enzymes to retain their enzymatic stability and activity. Essentially, the substrate can easily be entrapped by graphene with a high surface area and enter the inner space of microspheres through the quantities of rGO defects resulting in substrate's enrichment. Concentration of substrate and enzyme in this confined region will increase the chance of effective collisions between them (Xie et al. 2011).

3.6. Selectivity of the H_2O_2 biosensor

Selectivity is important in practical use of the biosensors. The selectivity of the biosensor is evaluated by using the amperometric current-time method among nine typical interfering substances (ascorbic acid (b), dopamine (c), uric acid (d), glucose (e), Mglycin(f), citric acid (g), acetic acid (h), MgSO₄ solutions (j) and NaCl solutions (j)). As seen in Fig. S10, nine tested substances do not interfere the result of testing H_2O_2 significantly. This result might be attributed to the intrinsic selectivity of Hb for its substances.

3.7. Stability and reproducibility of the biosensor

The stability of the Nafion/Hb/H-TiO₂-rGOMS/GCE is investigated through two methods. Firstly, 100 continuous cyclic scans on Nafion/Hb/H-TiO₂-rGOMS/GCE are repeated in the potential range from -0.7 V to 0.1 V at a scan rate of 0.1 V s⁻¹ (Fig. 6A). There is almost no decrease of the

1 voltammetric response and 96.3% of the initial current response was retained. Hence, the prepared
2 Nafion/Hb/H-TiO₂-rGOMS/GCE possesses excellent cycle stability. Secondly, the long-term stability
3 which is an important evaluation index for estimating the performance of biosensor is studied by
4 examining its current response during various storage periods. When not in use, the fabricated
5 biosensor is stored at 4 °C. The biosensor retained about 97.3% of its initial response to H₂O₂ after 30
6 days and 95.4% after 60 days, demonstrating good long-term stability (Fig. 6B). Additionally,
7 compared with that of Nafion/Hb/H-TiO₂-rGOMS/GCE, the stability of Nafion/Hb/TiO₂-rGO/GCE and
8 Nafion/Hb/TiO₂@rGOMS/GCE are relatively worse (Fig. S11). The good stability is probably
9 contributed to special hollow structure and excellent biocompatibility of prepared H-TiO₂-rGO MS
10 which can provide a favorable microenvironment for Hb to retain its bioactivity.

11 The reproducibility of the biosensor is investigated by determining 4 μM H₂O₂ in PBS (pH 7.0).
12 The relative standard deviation (RSD) is 2.4% respectively for 5 successive measurements. Five
13 biosensors prepared under the same conditions independently give a RSD of 2.7% for the
14 determination of 4 μM H₂O₂ in PBS (pH 7.0), indicating an acceptable electrode-to-electrode
15 reproducibility.

16 **3.8. Analysis of real samples**

17 In order to evaluate the analytical utility of the electrode in practice, the
18 Nafion/Hb/H-TiO₂-rGOMS/GCE is applied for determination of real contact lens care solution. Before
19 experiments, the real samples are diluted 5000 times with doubly distilled water and without any other
20 pretreatment. Recovery tests and KMnO₄ titration method were used to examine the reliability and
21 accuracy of this method. The H₂O₂ content of different samples and recoveries of added analyte are
22 evaluated, and the results confirm that determining the H₂O₂ concentration in real samples by using the

proposed method is reliable (Table S3).

4. Conclusion

Hollow TiO₂-rGO microspheres have been synthesized through a simple synthetic route, and subsequently used to fabricate a mediator-free biosensor for the detection of H₂O₂. The special hollow structure of microspheres can immobilize more Hb with biological activity, and the graphene can simply entrap the substrate. Hence, there is an increased chance of effective collisions between substrate and redox protein leading to improved performance of this sensor. More importantly, numerous TiO₂ nanoparticles with excellent biocompatibility on the microsphere may provide a protective microenvironment for Hb to retain its bioactivity and stability for long time. Due to the reasons above, the prepared biosensors display a low detection limit of 10 nM, a wide linear range of 0.1-360 μM for H₂O₂, especially an excellent long-term stability. This study indicates that hollow TiO₂-rGO microspheres can be used as biocompatible matrix for the immobilization of enzyme and the construction of direct electrochemical biosensor.

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6 **Figure Captions:**

7 **Fig. 1** Schematic illustration of the hollow TiO₂-rGO microsphere encapsulating hemoglobin and
 8 Nafion/Hb/TiO₂-rGO MS/GCE.

9 **Fig. 2.** Schematic illustration of synthesis steps for hollow TiO₂-rGO microspheres and
 10 corresponding SEM or TEM images. (A) Synthesis steps for hollow TiO₂-rGO microspheres. (B and C)
 11 SEM images of bare TiO₂ microspheres. (D and E) SEM images of TiO₂@GO microspheres. (F and G)
 12 TEM images of as-prepared hollow TiO₂-rGO microsphere.

13 **Fig. 3.** Cyclic voltammograms of Nafion/H-TiO₂-rGOMS/GCE (curve a), Nafion/Hb/GCE (curve
 14 b), Nafion/Hb/TiO₂/GCE (curve c), Nafion/Hb/rGO/GCE (curve d), Nafion/Hb/TiO₂-rGO/GCE (curve
 15 e), Nafion/Hb/TiO₂@rGOMS/GCE (curve f), Nafion/Hb/H-TiO₂-rGOMS/GCE (curve g) in 0.1M pH
 16 7.0 PBS at a scan rate of 0.1 V s⁻¹.

17 **Fig. 4.** Cyclic voltammograms of Nafion/Hb/H-TiO₂-rGOMS/GCE in 0.1 M pH 7.0 PBS
 18 containing 0 (a), 5 (b), 15 (c), 30 (d), 70 (e), 110 (f), 150 (g), 190 (h), 230 (i), 270 (j), 310 (k), 350 (l),
 19 390 (m) H₂O₂ with a scan rate of 0.1 V s⁻¹. Inset: plot of the cathodic peak current vs. H₂O₂

1 concentration: Nafion/Hb/H-TiO₂-rGOMS/GCE (curve a), Nafion/Hb/TiO₂@rGOMS/GCE (curve b),
2 Nafion/Hb/TiO₂-rGO/GCE (curve c).

3 **Fig. 5** (A) Typical current-time response of Nafion/Hb/H-TiO₂-rGOMS/GCE at -0.35V to
4 successive addition of H₂O₂ in stirred 0.1 M pH 7.0 PBS. (B) The steady state current vs. H₂O₂
5 concentration.

6 **Fig. 6.** (A) Cyclic voltammograms of Nafion/Hb/H-TiO₂-rGOMS/GCE in 0.1 M pH 7.0 PBS
7 containing 100 μ M H₂O₂ on the first day (a), 30th day (b) and 60th day (c) after the preparation. Scan
8 rate: 100 mV s⁻¹. Inset: The relationship between the cathodic peak current and the storage time. (B)
9 Cyclic voltammograms of Nafion/Hb/H-TiO₂-rGOMS/GCE in PBS (pH 7.0) with 100 continuous
10 cyclic scans. Scan rate: 100 mV s⁻¹. Inset: The relationship between I/I_0 and the cycle number. I and I_0
11 correspond to the cathodic peak current on any day and the first day after the preparation.

Figure 1

Fig. 1 Schematic illustration of the hollow TiO_2 -rGO microsphere encapsulating hemoglobin and Nafion/Hb/ TiO_2 -rGO MS/GCE.

Figure 2

Fig. 2 Schematic illustration of synthesis steps for hollow TiO_2 -rGO microspheres and corresponding SEM or TEM images. (A) Synthesis steps for hollow TiO_2 -rGO microspheres. (B and C) SEM images of bare TiO_2 microspheres. (D and E) SEM images of TiO_2 @GO microspheres. (F and G) TEM images of as-prepared hollow TiO_2 -rGO microsphere.

Figure 3

Fig 3 Cyclic voltammograms of Nafion/H-TiO₂-rGOMS/GCE (curve a), Nafion/Hb/GCE (curve b), Nafion/Hb/TiO₂/GCE (curve c), Nafion/Hb/rGO/GCE (curve d), Nafion/Hb/TiO₂-rGO/GCE (curve e), Nafion/Hb/TiO₂@rGOMS/GCE (curve f), Nafion/Hb/H-TiO₂-rGOMS/GCE (curve g) in 0.1M pH 7.0 PBS with a scan rate of 0.1 V s⁻¹.

Figure 4

- 1 **Fig. 4.** Cyclic voltammograms of Nafion/Hb/H-TiO₂-rGOMS/GCE in 0.1 M pH 7.0 PBS containing 0
- 2 (a), 5 (b), 15 (c), 30 (d), 70 (e), 110 (f), 150 (g), 190 (h), 230 (i), 270 (j), 310 (k), 350 (l), 390 (m) H₂O₂
- 3 with a scan rate of 0.1 V s⁻¹. Inset: plot of the cathodic peak current vs. H₂O₂ concentration:
- 4 Nafion/Hb/H-TiO₂-rGOMS/GCE (curve a), Nafion/Hb/TiO₂@rGOMS/GCE (curve b),
- 5 Nafion/Hb/TiO₂-rGO/GCE (curve c).

Figure 5

Fig. 5 (A) Typical current-time response of Nafion/Hb/H-TiO₂-rGOMS/GCE at -0.35V to successive addition of H₂O₂ in stirred 0.1 M pH 7.0 PBS. (B) The steady state current vs. H₂O₂ concentration.

Figure 6

Fig. 6 (A) Cyclic voltammograms of Nafion/Hb/H-TiO₂-rGOMS/GCE in PBS (pH 7.0) with 100 continuous cyclic scans. Scan rate: 100 mV s⁻¹. Inset: The relationship between I/I_0 and the cycle number. I and I_0 correspond to the cathodic peak current on any day and the first day after the preparation. **(B)** Cyclic voltammograms of Nafion/Hb/H-TiO₂-rGOMS/GCE in 0.1 M pH 7.0 PBS containing 100 μM H₂O₂ on the first day (a), 30th day (b) and 60th day (c) after the preparation. Scan rate: 100 mV s⁻¹. Inset: The relationship between the cathodic peak current and the storage time.