A Novel Nitrite Biosensor Based on Gold Dendrites with Egg White as Template

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Gold dendrites (AuD) were synthesized with egg white as the soft template and a novel nitrite (NO_2^-) biosensor was fabricated by assembly of a myoglobin (Mb)-L-cysteamine (Cys)-AuD biological hybrid. The results of Fourier transform infrared spectra and UV-visible spectra indicated that Mb retained its original structure in the resulting Mb-Cys-AuD. Electrochemical investigation of the biosensor showed a pair of well-defined, quasi-reversible redox peaks with $E_{\text{pa}} = -0.314$ V and $E_{\text{pc}} = -0.344$ V (vs. SCE) in 0.1 M, pH 7.0 sodium phosphate buffered saline at the scan rate of 200 mV/s. The transfer rate constant (k_s) was 1.49 s^{-1} . The Mb-Cys-AuD showed a good electrochemical catalytic response for the reduction of NO_2^- , with the linear range from 0.5 to 400 μM and the detection limit of 0.3 μM ($S/N = 3$). The apparent Michaelis-Menten constant (K_M^{app}) was estimated to be 0.2 mM. Therefore, the assembled bio-hybrid as a novel matrix opened up a further possibility for study on the design of enzymatic biosensors with potential applications.

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Introduction

Biocompatible materials, such as natural biomaterials, bio-hybrid, biopolymers and some nanomaterials, can provide a favorable microenvironment for redox proteins to realize their direct electrochemistry; and with them one can fabricate excellent biosensors.¹ Bio-hybrid materials can be associated with materials that have in common a biological component and an inorganic component.² While they can exhibit very different structures and unique properties mainly depending on the nature of the biological system. Bio-hybrid materials may have important advantages over conventional biomaterials including improved cellular integration, the potentially are more straightforward *in vivo* applicability, and they are able to effectively remodel their materials structure with time.³

Gold (Au) plays a special role in nanoscience and nanotechnology, due to the fact that Au is the most stable noble metal at the nano-scale. Thus, the designers of any nano-device requiring metallic components are likely to consider Au.⁴ The excellent biocompatibility, conductivity and catalytic properties also make Au candidate to amplify the electrode surfaces, to enhance the electron transfer between redox centers in proteins, and to act as catalyst to facilitate electrochemical reactions.⁵ Au offers unique surface chemistry properties that allow it to be used as a desired platform on which to self-assemble layers of organic molecules, usually bound to the gold by sulfur atoms.⁶ The abundant thiol and amino groups of biomaterials, make the fabrication of Au-associated bio-hybrids become a hot point for the development of novel biosensors.

Wet chemistry technique based on chemical reactions in solution is the most widely used procedure for the synthesis of

nano-Au with a wide range of sizes, shapes and dielectric environments. The average particle diameter and surface morphology, which are the most important parameters when applying the nanoparticles to biosensing, can be controlled experimentally in an easy manner by adjusting the preparation conditions.⁴ Conjugation of nano-Au with other nanomaterials and biomolecules is an attractive research area within nanobiotechnology.⁷ Peptide functionalized Au surfaces that connect the highly organized structure of a thiol self-assembled monolayers (SAMs) on Au with biologically relevant structural elements were prepared.⁸ Au of dendrimer-like structures (AuD) was synthesized by a one-pot route, wherein a novel surfactant solution was used as reducing agent and assembling agent.⁹ The dendritic structures have relatively large surface areas, which is beneficial for the immobilization of biomaterials.¹⁰ Here, a facile wet-chemical method for the synthesis of AuD was reported. Biomaterial egg white was used as the template, which provided two key elements of dendritic forms: the branching structures and the erratic winding travels of individual branches.

As well known, the major proteins of the egg white are ovalbumin, lysozyme, and ovotransferrin.¹¹ Thus, egg white can act as a macromolecular polymer,¹² and its excellent foaming and gelling properties¹³ allow it to be used as surfactant. The presence of egg white in the synthesis of nanomaterials leads to a different progression of the nano formation.¹¹ Using cheap, non-toxic and environmentally benign egg white protein, $\text{CuFe}_{2-x}\text{Cr}_x\text{O}_4$ ($0 \leq x \leq 1$) nanopowders were successfully synthesized with metal nitrates.¹⁴ Platelike CeO_2 nanoparticles was synthesized in an aqueous medium by a simple, cost-effective, and environmentally friendly method using cerium(III) acetate hydrate and freshly extracted egg white (ovalbumin).¹⁵ By using Co^{2+} and Co^{3+} salts, and freshly extracted ovalbumin, Co_3O_4 nanocrystals have been synthesized successfully.¹⁶ However, to our knowledge, there was no similar

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application in the synthesis of nano-Au based on egg white as the soft biotemplate reported in the literature.

The aim of the present work are to synthesize AuD by employing egg white as the soft biotemplate; and this material was employed to fabricate a nitrite (NO_2^-) biosensor by self-assembly technique. The electrochemical behavior of myoglobin (Mb) and its electrocatalysis towards nitrite (NO_2^-) in the proposed biosensor are further investigated.

Experimental

Reagents

Bovine myoglobin (Mb, M_w 17800), chloroauric acid tetrahydrate ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma (St. Louis, USA). Cys was from Bailingwei Co., Ltd. (Beijing, China). Eggs were obtained from the local market. A 0.1 M sodium phosphate buffered saline (PBS) with various pH values were prepared by mixing stock standard solutions of Na_2HPO_4 and KH_2PO_4 and adjusting the pH value with 0.1 M H_3PO_4 or NaOH.

Apparatus

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out on a CHI 660D electrochemical workstation (Shanghai CH Instrument Co. Ltd., China). A traditional three-electrode system was used with an Mb-modified electrode as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. Thus, all the potentials reported in this work had been measured *versus* SCE reference electrode. Fourier transform infrared (FTIR) spectra were recorded on KBr disk using a Nicolet 380 FTIR spectrometer (Thermo Electron Corp., USA). UV-visible spectra were obtained on a Cary 50 probe UV-visible spectrophotometer (Varian, Australia). Scanning electron microscopy (SEM) was done with a JSM-6700F scanning electron microscope (Japan Electron Company, Japan). All the tested solutions were purged with highly purified nitrogen for at least 20 min prior to a series of voltammetric experiments and were maintained under nitrogen atmosphere during the measurements. All the electrochemical experiments were conducted at room temperature ($25 \pm 2^\circ\text{C}$).

Preparation of the AuD

AuD was prepared as follows: 1 mL of HAuCl_4 solution (w/w, 1%) and 5 mL of freshly extracted egg white were added into 100 mL flask. Then, the solution was heated until boiling, and then 5 mL of sodium citrate solution (w/w, 1%) was slowly added, while stirring vigorously. There are large degeneration proteins floating at the surface of the solution and one should immediately filtrate. The wine red solution should be kept continuous boiling for 30 min; then, it should be allowed to cool down. The AuD was prepared and stored at 4°C for use. For controlled trials, conventional AuNPs were prepared as follows: 1 mL of HAuCl_4 solution (w/w, 1%) was added into 100 mL flask; then the mixture heated until the solution began boiling, next, 5 mL of sodium citrate solution (w/w, 1%) was quickly added while stirring vigorously. After continuous boiling for 30 min, the mixture was allowed to cool down. AuNPs were prepared.

Preparation of the Mb modified electrode

A DL-180 ultrasonic cleaning machine (35 kHz, Zhejiang Haitian Electron Instrument Factory, China) was used to dissolve

solutes and form homogeneous solutions. GCE of 3-mm diameter, before use, was first polished to mirror-like finish with 1.0, 0.3 and $0.05\ \mu\text{m}$ Al_2O_3 slurry on a polish cloth, and then rinsed with double-distilled water, then sonicated in ethanol and double-distilled water for 5 min, each. A $50\text{-}\mu\text{L}$ volume of AuD and $30\ \mu\text{L}$ of 0.1 M Cys were mixed for 30 min. Then, $50\ \mu\text{L}$ of 5 mM EDC and 5 mM NHS mixture was added into the solution, and $40\ \mu\text{L}$ of 20 mg/mL Mb was added; the mixture are shaken in a rocking incubator at $37 \pm 0.2^\circ\text{C}$ mixed for 24 h. Finally $10\ \mu\text{L}$ of the mixture was cast onto the surface of the bare GCE by using a syringe to prepare Mb-Cys-AuD/GCE. Without use, the biosensor was stored at 4°C in a refrigerator. For controlled trial, the Mb-Cys-AuNPs/GCE was fabricated with AuNPs other than AuD under the same process mentioned above; the Mb-Cys-AuD/GCE was prepared by *in situ* assembly as follows: $10\ \mu\text{L}$ of AuD was casted onto the surface of the bare GCE by using a syringe to prepare AuD/GCE; then, immersed into 0.1 M Cys for 30 min. The Cys-AuD/GCE was washed with pure water, then, $10\ \mu\text{L}$ of 4 mg/mL of Mb containing 5 mM EDC and 5 mM NHS in PBS was casted onto the surface of the Cys-AuD/GCE. Thus, *in situ* assembled Mb-Cys-AuD/GCE was prepared.

Results and Discussion

Strategy for the fabrication of Au and the Mb-Cys-AuD bio-hybrid

Diffusion limited aggregation processes produce complicated shapes qualitatively similar to real dendrites.¹⁷ The formatted Au nanoseeds at the origin of a lattice were self-assembled on the amino groups (NH_2) of the thermal denaturation protein through Au-NH bonds. Other Au nanoseeds were allowed to walk at random, just as diffusion from far away until they arrived at one of the lattice sites adjacent to the occupied site. Then they were stopped; another Au nanoseeds were launched and halted when adjacent to the two occupied sites; the sequence was repeated. An indefinitely large cluster may be formed in this way. A typical structure produced on a two-dimensional lattice is dendrimer-like nano Au.

Scheme 1 illustrates the general design strategy for the Mb-Cys-AuD bio-hybrid. Cys served as a linker between Mb and AuD. The self-assembly process of thiol groups (SH) at the terminal of Cys interacted with AuD to form Cys-AuD. The coupling reaction of NH_2 at the other terminal of Cys with carboxyl group of Mb strengthened the immobilization of Mb in the Mb-Cys-AuD. AuD formed as a tree, and Mb atoms were like apples on the tree. Cys worked as the carpopodium to keep Mb on AuD tightly. The strong interest in assembly based on SH with nano-Au is always due to the following aspects:¹⁸ (1) they can offer insulating barriers between an electrode and a redox couple to study the long-range electron transfer process; (2) they can fabricate supermolecule assemblies with tailored architecture and properties for creating selective voltammetric detectors and measuring very fast electron transfer kinetics. With the formation of SH-Au, Cys-AuD biocomposite, which provide a favorable matrix for the immobilization of Mb, were formed.

Characterization of the AuD and the Mb-Cys-AuD

The morphology of AuD observed by SEM is shown in Fig. 1. In Fig. 1A, AuD show the branching structures and the erratic winding travels of individual branches, which were in conformity with dendritic structure. SEM with high magnification showed a dotted pattern with diameters of 60 – 100 nm (as shown in Fig. 1B), proving that the dendrite consists of a single crystal,

Scheme 1 Scheme of the assembly process of the Mb-Cys-AuD/GCE.

Fig. 1 SEM images of AuD with low (A) and high (B) magnification.

Fig. 2 (A) FTIR spectra of Mb (a) and the Mb-Cys-AuD film (b). (B) UV-vis absorption spectra of 0.3 mg/mL Mb in PBS (a') and in the solution of Mb-Cys-AuD mixture (b'). The light path length, 1 cm.

not an agglomeration of small distinct nanoparticles.

FTIR spectra and UV-visible spectra were used to characterize the Mb-Cys-AuD. FTIR spectroscopy was used to monitor the secondary structure conformations of polypeptide chain of the Mb-Cys-AuD. In Mb, amide I band ($1700 - 1600 \text{ cm}^{-1}$) was caused by C=O stretching vibration of peptide linkages and amide II band ($1620 - 1500 \text{ cm}^{-1}$) was attributed to the combination of N-H in plane bending and C-N stretching vibrations of the peptide groups.¹⁹ If Mb is denatured, the

intensities and shape of amide I and II band will diminish or even disappear. As shown in Fig. 2A, the spectrum of free Mb (a) was similar to that of Mb in the Mb-Cys-AuD (b), which suggests that Mb retains the essential feature of its native structure in the Mb-Cys-AuD.

UV-visible Soret absorption band of Mb may provide some information on the conformational integrity of the protein and on the possible denaturation or the conformational change of heme region. Mb dissolved in PBS had the Soret band at

Fig. 3 (A) EIS plots for bare GCE (a), the Mb-Cys-AuD/GCE (b), and the AuD/GCE (c) in a solution of 1 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ + 0.1 M KCl as the supporting electrolyte. The frequencies were swept from 10^5 to 10^{-2} Hz. (B) CVs of the bare GCE (a'), Mb-Cys-AuD/GCE (b'), *in situ* assembled Mb-Cys-AuD/GCE (c') and Mb-Cys-AuNPs/GCE (d') in N_2 saturated PBS at the scan rate of 200 mV/s.

408.9 nm (a'), while in the Mb-Cys-AuD the Soret band was appeared at 409.2 nm (b') as shown in Fig. 2B. The difference of Mb and the Mb-Cys-AuD was less than 1 nm, indicating that Mb in the Mb-Cys-AuD had the microenvironment similar to the native state of Mb-heme in PBS.

Ferricyanide was chosen as a marker of electrochemical impedance spectroscopy (EIS) to further investigate the behaviors of the Mb-Cys-AuD. At the bare GCE, the redox process of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ probe showed an electron transfer resistance of about 182.0 Ω (curve a in Fig. 3A). When the GCE was modified with AuD (curve c in Fig. 3A), the diameter of the semicircle reduced to 110.5 Ω , suggesting that AuD has an excellent electrical conductivity. When Mb was immobilized on AuD (curve b in Fig. 3A), the electron transfer resistance was slightly increased to 120.8 Ω . Owing to the dielectric behavior of enzyme molecules for interfacial electron transfer processes, the higher enzyme coverage will result in a higher barrier for the interfacial electron transfer. These data confirmed that Mb had been successfully immobilized on AuD and that it blocked the electron exchange between the redox probe and the electrode.

In N_2 saturated 0.1 M PBS (pH 7.0), the bare GCE had no peaks (shown in Fig. 3B, curve a'). The *in situ* assembled Mb-Cys-AuD/GCE only showed one cathode peak at -0.409 V (shown in Fig. 3B curve c') due to the fact that, when AuD was casted on the electrode surface, some of the binding sites towards Cys were lost. A chain effect was shown. The assembly amount of Cys decreased, and sequentially, the amount of Mb attached to the proposed electrode was smaller than that of the assembled Mb-Cys-AuD bio-hybrid. Though a much bigger cathode peak at -0.329 was shown at the Mb-Cys-AuNPs/GCE, no anodic peak appeared. A pair of asymmetric redox peaks appeared in the Mb-Cys-AuD/GCE (Fig. 3B, curve b'), which was attributed to the redox process of heme $\text{Fe}(\text{III})/\text{Fe}(\text{II})$ couple. The above electrochemical performance of different electrodes indicated that AuD could provide a favorable microenvironment for Mb to retain its natural structure and to realize its direct electrochemistry. AuD plays important roles to facilitate the electron transfer from Mb the synergistic effect of Mb-Cys-AuD which is useful to amplify the signal output.

Direct electrochemistry of Mb

The direct electrochemistry of Mb in the Mb-Cys-AuD/GCE was studied by cyclic voltammetry (CV). Cyclic voltammograms (CVs) of Mb at the Mb-Cys-AuD/GCE with different scan rates

Fig. 4 CVs of the Mb-Cys-AuD/GCE in N_2 saturated PBS with different scan rates. From a to l: 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000 mV/s. Inset: the relationship between cathodic and anodic peak current with scan rate v .

are shown in Fig. 4. The modified electrode showed a pair of well-defined, quasi-reversible redox peaks with $E_{\text{pa}} = -0.314$ V and $E_{\text{pc}} = -0.344$ V (vs. SCE) in PBS (0.1 M, pH 7.0) with the formal potential $E^{0'} = -0.329$ V. The value of $E^{0'}$ was similar to Mb-mesopores cellular foams/GCE.²⁰ The peak-to-peak separation ΔE_{p} was 30 mV and about 1 ratio of cathodic to anodic current intensity at the scan rate of 200 mV/s, indicating that the direct electron transfer for Mb- $\text{Fe}(\text{III})/\text{Fe}(\text{II})$ was nearly reversible. The redox process of Mb at the Mb-Cys-AuD/GCE gave roughly symmetric anodic and cathodic peaks at relative slow scan rates. When the scan rate increased, the redox potentials (E_{pa} and E_{pc}) of Mb hardly shift. Meanwhile, the redox peak current changed linearly (inset, Fig. 4): $I_{\text{pa}} = 1.08 - 5.1 \times 10^{-3}v$, $r = 0.9891$; $I_{\text{pc}} = 0.21 - 5.7 \times 10^{-3}v$, $r = 0.9970$. This indicated that the electron transfer process for Mb at the Mb-Cys-AuD/GCE was a surface-confined mechanism in the above-mentioned potential range, manifesting the characteristics of a thin-layer surface-controlled electrochemical process.

Fig. 5 Influence of the pH value on the CVs of the Mb-Cys-AuD/GCE. pH values (from left to right): 4.0, 5.0, 6.0, 7.0, 8.0, 9.0. Inset: the relationship between $E^{\circ'}$ and pH. Scan rate, 500 mV/s.

The anodic and cathodic peak potentials were linearly dependent on the logarithm of the scan rates (n) with slopes of $-2.3RT/anF$ and $2.3RT/(1-a)nF$, respectively.¹ Hence, the charge-transfer coefficient a was calculated as 0.53. Heterogeneous electron transfer rate constant (k_s) was further estimated according to the following equation:²¹

$$\log k_s = a \log(1-a) + (1-a) \log a - \log(RT/nFv) - (1-a)aF\Delta E_p/(2.3RT) \quad (1)$$

Here, a is a charge-transfer coefficient. n the number of electron transfer. R , T and F symbols have their conventional meanings. ΔE_p is the peak to peak potential separation.

The result was 1.49 s^{-1} , which was higher than that 0.332 s^{-1} of the Nafion/Mb/multi-walled carbon nanotubes/carbon ionic liquid electrode (CILE)²² and 0.588 s^{-1} of the Co/CILE.²³ Thus, the Mb-Cys-AuD bio-hybrid can provide a favorable microenvironment for Mb to undergo a facile electron transfer reaction for two reasons: (1) the structure of AuD will promote effective immobilization of enzyme, protein and other bioactive substances. Indeed, the dendrites structure greatly increased the specific surface area and offered more binding sites for the immobilization of Mb; (2) the soft template egg white contains a lot of protein and amino acid, which provide a benign microenvironment for the assembly process.

The effect of pH value on the direct electrochemistry of Mb at the Mb-Cys-AuD/GCE was investigated. As shown in Fig. 5, the pH value had an obvious effect on the peak currents of the immobilized Mb. By comparison, a better electrochemical response of the immobilized Mb was obtained at pH 7.0. This is suggested that the neutral pH value is close to the physiological environment and could retain activity of the Mb effectively. Thus, all the experiments were performed in pH 7.0 PBS unless specially stated.

The peak potentials were also dependent on pH value. With the increase of pH value from 4.0 to 9.0, the formal potentials ($E^{\circ'}$) shifted linearly to the negative direction (Fig. 5 inset), and the linear regression equation was obtained as $E^{\circ'} (\text{mV}) = -0.8 - 47.8\text{pH}$, $r = 0.9757$ ($n = 5$) with a slope value of 47.8 mV/pH. The slope value was slightly less than the theoretically expected value of 59 mV/pH at 25°C. One reason might be the influence

Fig. 6 CVs of the obtained at the Mb-Cys-AuD/GCE in N_2 saturated PBS with 0.5, 85, 130, 260, 400 μM NO_2^- (from a to f), respectively. Inset: the relationship between cathodic peak current with the concentration of NO_2^- .

of the protonation states of ligands trans to the heme iron and amino acids around the heme, or the protonation of the water molecule coordinated to the central iron.²⁴ However, the above results still indicate that a single proton transfer accompanying one electron transfer happened in the electrochemical process of Mb at the Mb-Cys-AuD/GCE. The electron process can be expressed by the following equation:²⁵

Electrocatalysis of Mb to NO_2^-

The Mb-Cys-AuD/GCE exhibited good catalytic activity towards NO_2^- . When NO_2^- was added into PBS (0.1 M, pH 7.0), the catalytic reduction peak current at -0.369 V (Fig. 6, curve a) increased (curves b – f). Meanwhile, the oxidation peak current of Mb was decreased. One possible mechanism of electrocatalytic reduction of NO_2^- at Mb-based enzyme electrode is the following:²⁶

The cathodic peak current has a linear relationship with the concentration of NO_2^- in the range between 0.5 – 400 μM , as shown in the inset of Fig. 6, such a relationship which indicates the Mb-Cys-AuD/GCE can be used for the detection of NO_2^- . And the linear regression equation was $I_{\text{ss}} (\mu\text{A}) = 2.85 \times 10^{-2}C (\mu\text{M}) + 4.01$, with a correlation coefficient 0.9983 ($n = 20$). The detection limit is estimated to be 0.3 μM at a signal-to-noise ratio of 3. According to the Michaelis-Menten kinetic mechanism,

Table 1 Comparison of analytical performances for some NO₂⁻ biosensors

Biosensor	Linear range/ μ M	Detection limit/ μ M	K_M^{app} /mM	Ref.
Nafion/Mb/MWCNTs/CILE	8.0 – 196	6.0	11.1	23
HMS-GCE	8.0 – 216	0.8	0.72	29
Mb-ZnO-GE	10 – 180	4	—	30
{Pluronic/Mb} ₈ -GCE	10 – 6000	5	—	31
Nafion/SLGnP-TPA-Mb/GCE	50 – 2500	10	—	32
GNRs@SiO ₂ -Mb/RTIL-solgel/GCE	40 – 5000	20	10.5	33
Nafion-BMIMPF ₆ /Mb/CILE	100 – 8400	50	1.46	34
Mb-Cys-AuD/GCE	0.5 – 400	0.3	0.2	This work

MWCNTs, multi-walled carbon nano-tubes; HMS, hexagonal mesoporous silica; ZnO, zinc oxide; GE, graphite electrode; Pluronic, poly(ethylene oxide)₁₀₀-poly(propylene oxide)₆₅-poly(ethylene oxide)₁₀₀ triblock copolymer; SLGnP, single-layer graphene nanoplatelet; TPA, tetrasodium 1,3,6,8-pyrenetetrasulfonic acid; GNRs@SiO₂, silica-coated gold nanorods; RTIL, room temperature ionic liquid; BMIMPF₆, 1-butyl-3-methylimidazolium hexafluorophosphate.

the apparent Michaelis-Menten constant K_M^{app} can be obtained from the electrochemical version of the Lineweaver-Burk equation:^{27,28}

$$1/I_{\text{ss}} = 1/I_{\text{max}} + K_M^{\text{app}}/(I_{\text{max}}C) \quad (8)$$

Here I_{ss} is the diffusion limiting current after the addition of the substrate. C is the bulk concentration of the substrate, and I_{max} the maximum current measured under the saturate substrate conditions. The value of K_M^{app} and I_{max} can be obtained by the slope and the intercept of the plot of the reciprocals of the steady-state current *versus* NO₂⁻ concentration. The apparent Michaelis-Menten constant K_M^{app} value was calculated to be 0.2 mM, which was much lower than the values in some previous reports. It is well-known that a low value of K_M^{app} represents a higher biological affinity of Mb in catalytic reaction of NO₂⁻. And, Mb can retain higher activity in the Mb-Cys-AuD. In Table 1, some of the analytical characteristics obtained in this work are compared with those previously reported in the literature.^{23,29-34} It can be seen that the responses of the Mb-Cys-AuD/GCE are, in most cases, superior, especially the linear range and the detection limit, to the values obtained previously using the NO₂⁻ biosensor.

Repeatability and stability of the NO₂⁻ biosensor

The stability and repeatability of the biosensor based on the Mb-Cys-AuD/GCE were studied in the linear range. The relative standard deviation (RSD) was 2.3% for seven successive measurements of 30 μ M O₂⁻ in PBS; such values show that the proposed biosensor possessed a good repeatability. In repetitive potential scan circles, CV responses of the biosensor in 0.1 M pH 7.0 N₂ saturated PBS containing 100 μ M NO₂⁻ showed a little decrease (2 – 3%). After that, the CV responses remain stable and the biosensor retained above 95% of its initial response after 20 cycles, indicating that the biosensor was stable. The storage stability of the biosensor was further investigated. The cathodic peak current of the Mb-Cys-AuD/GCE was measured using the same electrode and it retained above 95% of its initial response when after 4 weeks

Table 2 Detection results of NO₂⁻ in waste water sample

Sample No.	Content/ μ M	Added/ μ M	Found/ μ M	Recovery, %	RSD, %
1	2.5	30	32.7	100.6	2.3
2	4.1	30	34.0	99.7	3.1
3	5.3	30	35.6	100.8	2.9

The results are of average of five determinations by the biosensor.

stored at 4°C. These results displayed how good the stability of the biosensor based on the Mb-Cys-AuD/GCE was.

Determination of NO₂⁻ in real samples

In order to verify the reliability of the modified electrode, we checked the NO₂⁻ concentration in area 1 sample of waste water. Recovery studies were also carried out in waste water samples by using the standard addition method for the controlled trial. The determination results are summarized in Table 2. The results were in acceptable agreement and thus the modified electrode could be used for the NO₂⁻ sample analysis.

Conclusions

In this research, Mb was immobilized effectively into the assembled Mb-Cys-AuD bio-hybrid and a novel NO₂⁻ biosensor was successfully fabricated based on the bio-hybrid with GCE as the basal electrode. The biosensor exhibited a variety of good electrochemical characteristics, including low detection limit, high catalytic ability, wide linearity and a larger electron transfer rate constant of 1.49 s⁻¹. These advantages should be attributed to the following: (1) AuD plays an important role in facilitating the electron transfer between Mb and the electrode surface. The arrangement and uneven vent length structure of the AuD not only made Mb effectively immobilize, but also the AuD could be inserted into the structure of Mb as nano wire for electron transfer; (2) The self-assembly process of SH of the Cys with AuD, followed by a coupling reaction between Cys with Mb for the fabrication of the biosensor, provides a good platform for the direct electrochemistry of Mb and NO₂⁻ biosensing. The synergistic effects of Mb-Cys-AuD not only improved the electron transfer ability of our system, but also exhibited the signal amplification of nanosize materials. The present study has introduced the combination of biomaterial with syntheses of novel-shaped nanomaterials for the study of redox proteins by electrochemical methods, and such a combination offers new designs of enzymatic biosensors with potential applications in direct electrochemistry and biocatalysis. Further works derived by employing this strategy for the fabrication of novel biosensors are on our schedule.

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References

1. X. J. Zhao, Z. B. Mai, X. H. Kang, Z. Dai, and X. Y. Zou, *Electrochim. Acta*, **2008**, 53, 4732.
2. C. Sicard, R. Brayner, J. Margueritat, M. Hémadi, A. Couté, C. Y. Mian, C. Hakib Djediat, J. Aubard, F. Fiévet, J. Livage, and T. Coradin, *J. Mater. Chem.*, **2010**, 20, 9342.
3. M. Yoshida, K. H. Roh, S. Mandal, S. Bhaskar, D. Lim, H. Nandivada, X. Deng, and J. Lahann, *Adv. Mater.*, **2009**, 21, 4920.
4. J. Shan and H. Tenhu, *Chem. Commun.*, **2007**, 44, 4580.
5. Y. Li, H. J. Schluesener, and S. Q. Xu, *Gold Bull.*, **2010**, 43, 29.
6. P. Yáñez-Sedeño and J. M. Pingarrón, *Anal. Bioanal. Chem.*, **2005**, 382, 884.
7. E. Katz and I. Willner, *Angew. Chem., Int. Edit.*, **2004**, 43, 6042.
8. I. F. Gallardo and L. J. Webb, *Langmuir*, **2010**, 26, 18959.
9. S. F. Pang, T. Kondo, and T. Kawai, *Chem. Mater.*, **2005**, 17, 3636.
10. C. Kim, J. G. Oh, Y. T. Kim, H. Kim, and H. Lee, *Electrochem. Commun.*, **2010**, 12, 1596.
11. V. Pipich, M. Balz, S. E. Wolf, W. Tremel, and D. Schwahn, *J. Am. Chem. Soc.*, **2008**, 130, 6879.
12. A. E. Sommer, G. Fetter, P. Bosch, and O. Novelo, *Polym. Adv. Technol.*, **2011**, 22, 2638.
13. V. Lechevalier, R. Jeantet, A. Arhaliass, J. Legrand, and F. Nau, *J. Food Eng.*, **2007**, 83, 404.
14. M. A. Gabal, *Mater. Lett.*, **2010**, 64, 1887.
15. S. Maensiri, C. Masingboon, P. Laokul, W. Jareonboon, V. Promarak, P. L. Anderson, and S. Seraphin, *Cryst. Growth Des.*, **2007**, 7, 5.
16. T. O. Ahmadov, Z. Durmus, A. Baykal, and H. Kavas, *Chem. Mater. Sci.*, **2011**, 47, 426.
17. T. A. Witten, *Phys. Rev. B*, **1983**, 27, 5686.
18. S. L. Yang, B. F. Xu, J. Q. Zhang, X. D. Huang, J. S. Ye, and C. Z. Yu, *J. Phys. Chem. C*, **2010**, 114, 4389.
19. W. Sun, X. Q. Li, Y. Wang, X. Li, C. Z. Zhao, and K. Jiao, *Bioelectrochemistry*, **2009**, 75, 170.
20. L. Zhang, Q. Zhang, and J. H. Li, *Biosens. Bioelectron.*, **2010**, 26, 846.
21. E. Laviron, *J. Electroanal. Chem.*, **1979**, 101, 19.
22. W. Sun, X. Q. Li, Y. Wang, X. Li, C. Z. Zhao, and K. Jiao, *Bioelectrochemistry*, **2009**, 75, 170.
23. W. Sun, X. Q. Li, P. Qin, and K. Jiao, *J. Phys. Chem. C*, **2009**, 113, 11294.
24. C. Y. Liu and J. M. Hu, *Biosens. Bioelectron.*, **2009**, 24, 2149.
25. X. Q. Liu, H. J. Li, F. Wang, S. Y. Zhu, Y. L. Wang, and G. B. Xu, *Biosens. Bioelectron.*, **2010**, 25, 2194.
26. W. J. Shan, P. L. He, and N. F. Hu, *Electrochim. Acta*, **2005**, 51, 432.
27. R. A. Kamin and G. S. Wilson, *Anal. Chem.*, **1980**, 52, 1198.
28. F. R. Shu and G. S. Wilson, *Anal. Chem.*, **1976**, 48, 1679.
29. Z. H. Dai, X. X. Xu, and H. X. Ju, *Anal. Biochem.*, **2004**, 332, 23.
30. G. Zhao, J. J. Xu, and H. Y. Chen, *Anal. Biochem.*, **2006**, 350, 145.
31. Y. Xie, N. F. Hu, and H. Y. Liu, *J. Electroanal. Chem.*, **2009**, 630, 63.
32. R. Yue, Q. Lu, and Y. K. Zhou, *Biosens. Bioelectron.*, **2011**, 26, 4436.
33. W. L. Zhu, Y. Zhou, and J. R. Zhang, *Talanta*, **2009**, 80, 224.
34. W. Sun, X. Q. Li, and K. Jiao, *Electroanalysis*, **2009**, 21, 959.