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Self-assembled film of hydrophobins on gold surfaces and its application to electrochemical biosensing

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ABSTRACT

Hydrophobins are small fungal proteins which self-assemble on interfaces and significantly change the surface wettability. The self-assembled film of hydrophobin HFBI on a gold surface improved the surface hydrophilicity with water contact angle changing from $73.8 \pm 1.8^\circ$ to $45.3 \pm 1.4^\circ$. A quartz crystal microbalance (QCM) analysis indicated that the HFBI coverage density on a gold surface was 588 ng cm^{-2}, and the self-assembled film remained stable under different pH values ranging from 1 to 13. A hydrophilic protein such as choline oxidase (ChOx) was then successfully immobilized on the HFBI modified gold surface. To evaluate the bioactivity of immobilized enzyme, an amperometric choline biosensor was constructed based on the Gold/HFBI/ChOx electrode, which produced as large as 4578.27 nA response current by 0.238 μg immobilized ChOx, when saturated by choline substrate. Comparing with our choline biosensors previously reported, the HFBI self-assembled film exhibited excellent capability to preserve the bioactivity of ChOx, hence a great potential in electrochemical biosensing is suggested.

1. Introduction

In the study of fungal biology, interesting phenomena have been observed that cell wall surfaces of the hypha growing in a moist substrate are hydrophilic, while the surfaces of aerial hyphae and airborne spores are hydrophobic. Genetic investigations revealed that the adjusting of fungal cell wall surface wettability is realized through secretion of hydrophobins, which are a family of small proteins produced exclusively by filamentous fungi [1,2]. Hydrophobins help fungi fulfill a series of interface related biological functions, such as mediating adhesion of fungal structures, lowering of water surface tension, and formation of coatings [3,4].

Firstly named by Wessels and co-workers in 1991 [1], hydrophobins are found easy to self-assemble at various interfaces to form robust and orderly membranes, which reverse the wettability of surfaces [5,6]. Structural analysis indicates that a hydrophobin consists of approximately 100 amino acid residues with a characteristic pattern of eight cysteine residues forming four disulfide bonds [7]. Their structural fold allows the display of a large flat hydrophobic patch by utilizing aliphatic side chains located near the loop regions of two adjoining hairpins [2]. Therefore one side of the protein consists solely of hydrophobic aliphatic side chains that form a planar hydrophobic patch on the otherwise mostly hydrophilic protein surface. With this unique amphiphatic structure, hydrophobins are able to change a hydrophobic surface into hydrophilic and vice versa [4].

Recently, hydrophobin modification on various kinds of surfaces such as Teflon, Nafion, Lycra [6], glass [8], mica, PDMS [9], and glassy carbon electrode [10–14] has been reported, which has greatly changed the surface wettability. This remarkable surface activity of hydrophobins has implied great prospects in biofunctionalization of many hydrophobic surfaces. Since biomolecules are mostly hydrophilic, highly hydrophilic surfaces are required in their immobilization. The amphiphilic structure helps hydrophobins to easily self-assemble on relatively hydrophobic surfaces and improve the surface hydrophilicity, which would facilitate subsequent biomolecular immobilization. Furthermore, with high biocompatibility [15,16], hydrophobin matrix could well preserve the bioactivities of immobilized biomolecules and may serve as a suitable biomoleculer immobilizing biomaterial.

We are interested in immobilizing redox enzymes on gold surfaces via hydrophobin self-assembled film. Redox enzymes catalyze the electron transfer from electron donors to electron acceptors, which can be quantitatively recorded by electrochemical measurements. A 7.5-kDa hydrophobin, HFBI from *Trichoderma reesei*, has been used in this work to immobilize choline oxidase (ChOx). An amperometric choline biosensor has been constructed based on

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the HFBI and ChOx modified gold electrode, to evaluate the performance of hydrophobin in protein immobilization.

Gold is an excellent electric conductor with fine ductility and chemical inertness, thus an ideal choice for developing bioelectronic devices. So far, electrochemical applications of hydrophobins have been carried out using hydrophobic glassy carbon electrodes [10–14], while hydrophobin modified gold surfaces has not yet been investigated. In this work, HFBI modification on smooth gold surfaces has been proven to effectively enhance the surface hydrophilicity and immobilize ChOx with its bioactive conformation. As a result, this new attempt may provide a promising approach to surface functionalizing in electrochemical biosensing.

2. Materials and methods

2.1. Reagents

Hydrophobin from *T. reesei* (HFBI, p*I* 5.7, in 0.1 M NaCl ≥98% by agarose gel electrophoresis, lyophilized powder containing 22.3 wt.% HFBI) was isolated and purified as previously reported [9]. Choline oxidase from *Alcaligenes species* (ChOx, EC1.1.3.17, p*I* 5.6, 17 units/mg protein), bovine serum albumin (BSA, pH ~ 7, 1% in 0.15 M NaCl ≥98% by agarose gel electrophoresis, lyophilized powder), and aluminum oxide nanopowder were purchased from Sigma–Aldrich Co., Choline chloride was purchased from Shanghai SSS Reagent Co. (China). Freshly prepared 0.1 M phosphate buffer (pH 6.8) consisting of Na_2HPO_4 and KH_2PO_4 was used to dissolve proteins and employed as supporting electrolyte. Solutions of different pH values ranged from 1 to 13 were obtained by adjusting phosphate buffer with HCl or NaOH. All the chemical reagents were of analytical grade and dissolved with deionized distilled water. All experiments were performed at room temperature, approximately 25 °C.

2.2. Characterization of self-assembled film

Gold-coated slides were prepared by gold sputtering onto glass substrates, at 35 mA current for 180 s by SCD 005 Sputter Coater (BAL-TEC Co., Switzerland). Clean and smooth gold slides were placed in small culture dishes and immersed in 20 μg/mL HFBI or 200 μg/mL BSA solutions for 30 min. Some of the immersed slides were air dried overnight in the culture dishes to deposit protein coatings on gold surfaces, while the others were rinsed by 100 mL flow phosphate buffer for 1 min, to wash away weakly adsorbed proteins before air drying. Surface wettability of the pretreated gold slides was tested by water contact angle measurement using a contact angle analyzer (Model G1023-MK3, Krüss GmbH, Germany).

A quartz crystal microbalance (QCM, Model QCA917 with a software Winchem, SEIKO EG&G Co.) was used to monitor the deposition of protein films and the film stability against acid and alkaline conditions. The sensor composed of an AT-cut 9 MHz piezoelectric quartz resonator with gold thin layers (effective surface area: 0.19 ± 0.01 cm^2) deposited on its two faces is excited to oscillation in the thickness shear mode at its fundamental resonant frequency, by applying a RF voltage across the electrodes near the resonant frequency. Protein concentrations for QCM measurement were 20 μg/mL for HFBI and 2 mg/mL for ChOx.

2.3. Electrodes modification with proteins

Gold electrodes (3 mm diameter) were polished with an aluminum oxide nanopowder, and then washed ultrasonically in ethanol and deionized distilled water, each for 30 min. The pretreated electrodes were immersed in 20 μg/mL HFBI solutions for 30 min and rinsed in pH 6.8 phosphate buffer for 10 min without drying-procedure. Then electrodes were transferred into 2 mg/mL ChOx solution for 30 min, followed by a 10-min-rinsing in pH 6.8 phosphate buffer. The modified electrodes were stored in pH 6.8 phosphate buffer at 4 °C when not in use.

2.4. Electrochemical measurements

Electrochemical measurements were carried out by a Potentiostat–Galvanostat (Model 283 with a software M270, EG&G Co.). A conventional three-electrode system comprising the modified gold electrode as working electrode, a Pt wire (1 mm diameter) as counter electrode and an Ag/AgCl (saturated KCl) as reference electrode was employed for all electrochemical experiments in an electrochemical cell filled with 20 mL pH 6.8 phosphate buffer. In cyclic voltammetry (CV) measurements, the scan rate was set at 50 mV/s. In steady-state amperometric measurements, the potential was set at +400 mV under gently magnetic stirring.

3. Results and discussion

3.1. Wettability of HFBI modified gold surfaces

Water contact angle was measured to evaluate the wettability of gold surfaces before and after HFBI modification. As shown in Table 1, the unmodified bare gold surface exhibited a weak hydrophilicity, while the surface hydrophilicity was remarkably improved after HFBI processing. This amelioration of surface wettability could be realized at an HFBI concentration as low as 20 μg/mL, without being affected by the presence of washing procedure.

HFBI molecules formed robust films on gold surfaces and changed the surface wettability more steadily, as compared with bovine serum albumin (BSA), a widely used non-specific blocking protein and protective protein in biochip and biosensor research, which was documented with easy physical adsorption on both hydrophilic and hydrophobic surfaces [17–20]. When used at a concentration of 200 μg/mL without washing procedure after protein coating, BSA could also abundantly adsorb on a bare gold surface and apparently change the surface wettability, which was even slightly more hydrophilic than the HFBI modified gold surface. However, this kind of physically adsorbed BSA coating was not stable. Once washed by flow phosphate buffer, the BSA protein would dissociate from the surface, which hence returned to be a weakly hydrophilic surface.

3.2. QCM analysis of HFBI self-assembled film

A self-assembly of HFBI film on a gold surface and subsequent immobilization of ChOx was monitored by QCM. Relationship between the decrease in resonant frequency (Δf) and the adsorbed mass (Δm) was given by the Sauerbrey equation [21], according to which 1 Hz frequency change of the quartz crystal used in this work corresponded to a mass increase of 5.45 ng cm^{-2}.

Table 1
Wettability of differently prepared gold surfaces.

	Water contact angle[a]
Bare gold surface	73.8 ± 1.8°
HFBI coating[b]	43.1 ± 1.2°
HFBI treatment[c]	45.3 ± 1.4°
BSA coating[b]	37.8 ± 1.9°
BSA treatment[c]	66.7 ± 2.3°

[a] Average value ± standard deviation (n = 5).

[b] Protein coating was prepared without wash after immersing in protein solution, thus the surface was covered with the remained solutes during air dry procedure.

[c] Protein treatment was followed by a flow phosphate buffer wash after immersing in protein solution, thus the surface could not get modified unless a stable film was formed on the surface.

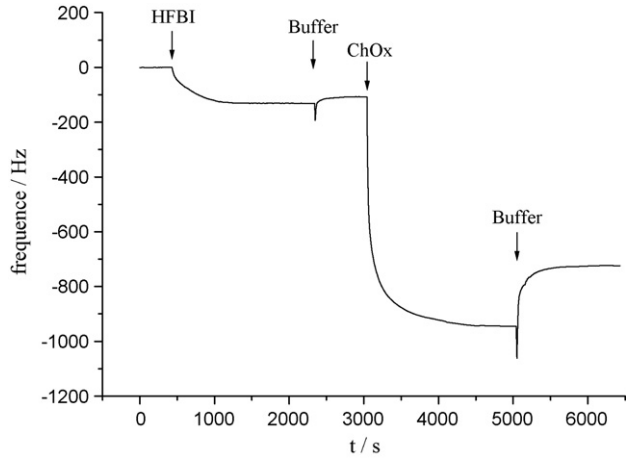

Fig. 1. Frequency changes of quartz crystal microbalance during sequential HFBI self-assembly and ChOx immobilization on a gold surface. The arrows indicated the removing of existing solutions and injection of new solutions: 20 μg/mL HFBI, pH 6.8 phosphate buffer, 2 mg/mL ChOx, and pH 6.8 phosphate buffer in turn.

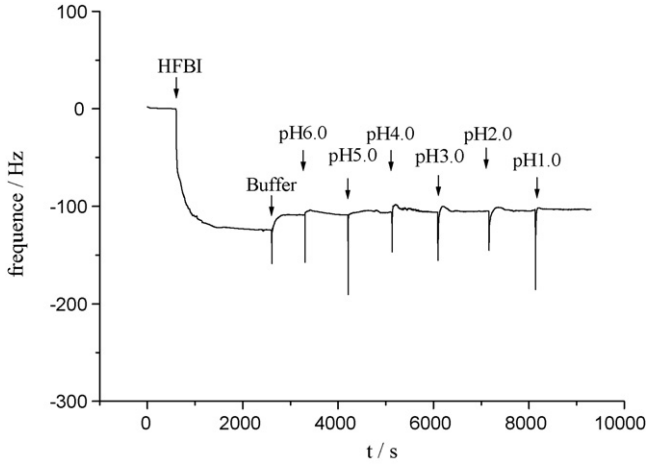

Fig. 2. Stability of HFBI self-assembled film on a gold surface under acid conditions, examined by quartz crystal microbalance. The arrows indicated the removing of existing solutions and injection of new solutions: 20 μg/mL HFBI, pH 6.8 phosphate buffer, and different acid solutions in turn.

Fig. 1 indicated that HFBI rapidly adsorbed on the gold surface and a small amount of weakly adsorbed HFBI was removed by the following phosphate buffer rinse. Then ChOx was successfully immobilized on the HFBI modified surface. The average frequency shifts were -107.9 ± 0.7 Hz for HFBI and -617.7 ± 1.9 Hz for ChOx, with relative standard deviation (R.S.D.) as 0.65% and 0.31%, respectively. According to the Sauerbrey equation, the masses deposited on the gold surface were calculated to be 588 ng cm^{-2} for HFBI and 3366 ng cm^{-2} for ChOx.

Its ineludible to mention that the self-assembly of HFBI on a gold surface is stronger than on a platinum surface. We previously reported [22] in the fabrication of an amperometric glucose biosensor that the masses of HFBI self-assembled on platinum surfaces were 306 ng cm^{-2} at pH 5.1 and 273 ng cm^{-2} at pH 6.8, either of which was only about one half of the mass of HFBI self-assembled on the gold surface. While immobilizing ChOx on the HFBI modified platinum surface, a maximum enzyme quantity as much as 744 ng cm^{-2} could be reached (figure not shown), which was less than a quarter of the ChOx quantity immobilized on the HFBI modified gold surface. Although the two noble metals shared quite a lot of similarities in physical and chemical characteristics as widely applied biosensing substrates, the results of QCM analysis suggested that HFBI had different self-assembly structures on gold and platinum surfaces, specific micro-organizations of which remained unclear.

The globular, soluble form of HFBI is a rigid round molecular with its diameter being 2–3 nm [4], and the area of planar hydrophobic patch being 4 nm^2 [23]. As a result, compact monolayer of HFBI on a hydrophobic surface would have a surface coverage density about 311.5 ng cm^{-2}, considering the molecular weight of HFBI is 7.5 kDa. Similar experimental results of surface coverage density for several HFBI monolayers have been reported, respectively 243 ng cm^{-2}, 416 ng cm^{-2} and 331 ng cm^{-2} [2]. The micro-structures of hydrophobin multilayers remain unclear, while greatly depending on the manners of film preparation and specific surfaces. Since the surface coverage density of HFBI on a gold surface was 588 ng cm^{-2}, the HFBI film self-assembled on a gold surface might be composed of complex structure mixed by monolayer and bilayer, whose thickness would be around 2–6 nm.

QCM was also used to examine the stability of the HFBI self-assembled film under different pH values. As shown in Figs. 2 and 3, the HFBI film exhibited extremely high stability in both acid and alkaline conditions. During the pH change from 1.0 to 13, not more than 5% of the total amount of HFBI detached from the surface.

3.3. CV study on the ChOx bioactivity

QCM results demonstrated that ChOx was successfully immobilized via HFBI self-assembled film on a gold surface, while the bioactivity of immobilized ChOx still needed further verification. ChOx was a flavoprotein catalyzing the conversion of choline into betaine via betaine aldehyde with oxygen as electron acceptor, as follows [24].

$$\text{Choline} + 2O_2 + H_2O = \text{betaine} + 2H_2O_2 (\text{overall reaction})$$
$$\text{Choline} + O_2 = \text{betaine aldehyde} + H_2O_2 \quad (a)$$

$$\text{Betaine aldehyde} + O_2 + H_2O = \text{betaine} + H_2O_2 \quad (b)$$

It is possible to detect the extent of reaction by electrochemical measurements of H_2O_2 produced in the reaction, thereby to evaluate the catalytic activity of immobilized ChOx.

Gold electrode was modified successively by HFBI and ChOx, and then utilized as working electrode in the CV measurements to check the ChOx activity. A typical redox peak of H_2O_2 was observed around +400 mV (Fig. 4a), suggesting that ChOx catalyzed the oxidation reaction of choline and produced H_2O_2. No evidence of such reaction was observed under circumstances without ChOx (Fig. 4c) or without choline (Fig. 4d). With an electrode surface

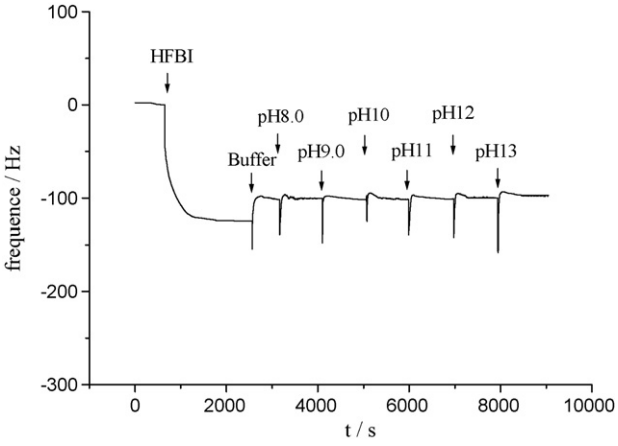

Fig. 3. Stability of HFBI self-assembled film on a gold surface under alkaline conditions, examined by quartz crystal microbalance. The arrows indicated the removing of existing solutions and injection of new solutions: 20 μg/mL HFBI, pH 6.8 phosphate buffer, and different alkaline solutions in turn.

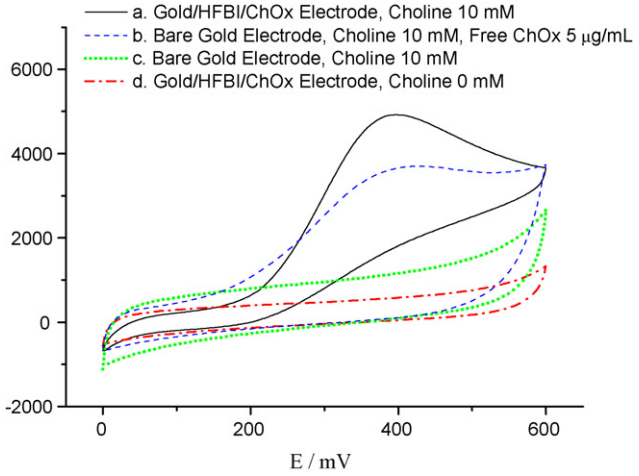

Fig. 4. Cyclic voltammograms of Gold/HFBI/ChOx electrode (a and d) in the presence of 10 mM choline (a) and 0 mM choline (d), compared with unmodified bare gold electrodes (b and c) in the presence of 10 mM choline (b and c) and 5 μg/mL free ChOx (b). Measurements were taken in 20 mL pH 6.8 phosphate buffer at room temperature, scan rate 50 mV/s.

area of 0.0707 cm^2, the quantity of ChOx immobilized on the electrode would be 0.238 μg, according to the QCM results. Compared with 100 μg free enzyme dispersed in the supporting electrolyte (Fig. 4b), the immobilized ChOx on electrode showed greater current response to 10 mM choline, because the immobilized ChOx was confined to the electrode surface and the electrons transferred during the reaction was able to be promptly passed to the current circuit.

3.4. Amperometric response of the choline biosensor

Based on the HFBI and ChOx modified gold electrode, an amperometric choline biosensor was constructed, which exhibited rapid and sensitive current response to successively added choline at +400 mV working potential, with response time no longer than 5 s.

Fig. 5 indicated the amperometric response to choline, representing the velocity of the enzymatic reaction. It well accorded with the kinetics of typical enzymatic reaction, described by

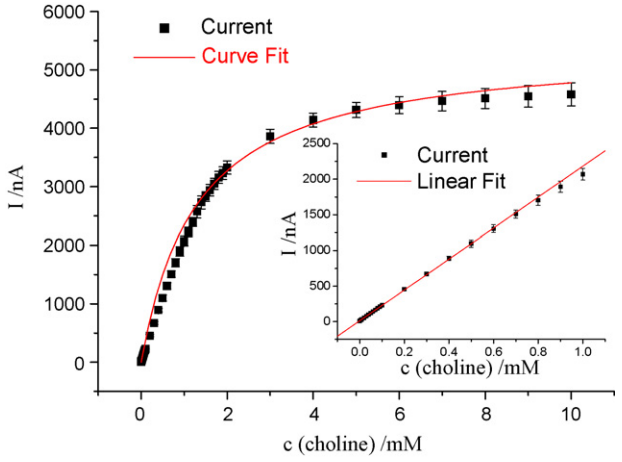

Fig. 5. Amperometric responses of Gold/HFBI/ChOx electrodes to choline. Measurements were taken in 20 mL pH 6.8 phosphate buffer at room temperature, with working potential at +400 mV. Error bars indicated the standard deviations ($n = 5$). Curve fit of $Y = V_{max} \times X/(K_m + X)$ was shown in red curve. Inset displayed detailed data within linear range (0.01–1.0 mM), with linear fit ($R = 0.9996$, $p < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

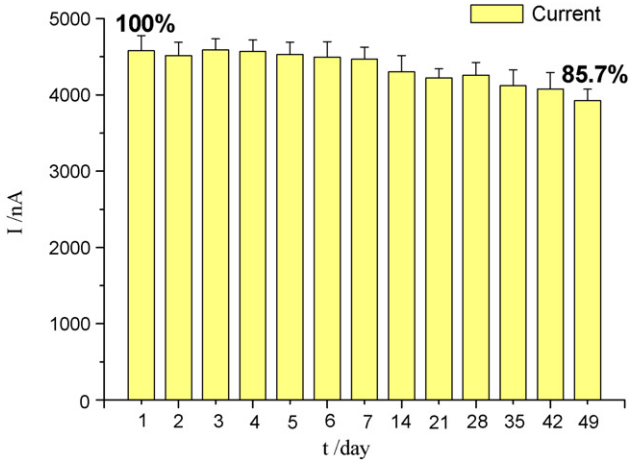

Fig. 6. Stability of Gold/HFBI/ChOx electrodes stored in pH 6.8 phosphate buffer at 4 °C during 7 weeks. Measurements were taken in 20 mL pH 6.8 phosphate buffer containing 10 mM choline at room temperature, with working potential at +400 mV. Error bars indicated the standard deviations ($n = 5$).

Michaelis–Menten equation [25]:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where V was the reaction velocity (represented by Iss, the steady-state current after adding substrate), V_{max} was the maximum reaction velocity (represented by I_{max}, the maximum current measured under saturated substrate condition), Michaelis constant (K_m) was the substrate concentration that lead to half-maximum velocity, and [S] was the bulk concentration of the substrate.

The apparent Michaelis constant (K_m^{app}) of immobilized ChOx on the Gold/HFBI/ChOx electrode was calculated to be 1.30 mM, which was close to the literature value of free ChOx (0.87 mM) [26]. The slightly higher K_m^{app} value suggested depressed affinity to choline, probably due to certain changes of the protein conformation after immobilization. On the other hand, the enzymatic activity was preserved in good condition, judging from the fairly high I_{max} and long life time of the enzyme electrode.

The most highlighted feature of HFBI self-assembled film on gold surfaces was that it could significantly keep the bioactivity of immobilized enzyme. The current response to 10 mM choline reached 4578.27 nA when 0.238 μg ChOx was immobilized on the HFBI modified gold electrode. For another choline biosensor we previously reported [27], the current response to 10 mM choline was only 2063.74 nA when 40 μg ChOx was immobilized into a sol–gel silicate film on the multi-wall carbon nanotubes modified platinum electrode. The highly effective biocatalytic reaction of a slight quantity of bimolecular would be useful in electrochemical biosensing, especially when the target bimolecular were precious and difficult to be obtained with large amount, or when extremely thin film was required during the development of micro-sized bioelectronic devices.

3.5. Storage stability of the choline biosensor

To examine the long-term storage stability, the Gold/HFBI/ChOx electrodes were used for periodic detection of 10 mM choline during 7 weeks, while stored in pH 6.8 phosphate buffer at 4 °C after use. As shown in Fig. 6, the Gold/HFBI/ChOx electrodes suffered a gradual and slight bioactivity loss, but 85.7% of its initial current response remained after 7 weeks storage. In contrast, the Pt/MWNT/ChOx electrode we reported previously [27], which produced a maximum 2063.74 nA current by 40 μg ChOx, remained 75.7% of its initial current responses after 1 month storage. The storage stability

of the Gold/HFBI/ChOx electrode turned out to be quite excellent, which suggested that the self-assembled film of HFBI successfully preserved the bioactivity of ChOx during a long life time.

4. Conclusions

Here we have reported HFBI self-assembled film on gold surfaces as a new biofunctionalizing matrix. The robust and stable hydrophobin film distinctly improves the surface hydrophilicity to facilitate protein immobilization, and preserved the bioactivity of immobilized protein. Therefore it would be a quite promising biomaterial in the field of electrochemical biosensing.

At the same time, an amperometric choline biosensor has been constructed based on the HFBI and ChOx modified gold electrode, which exhibits potential in research of mammalian nervous system [28] and detection of cholinesterase inhibitors such as organophosphate pesticides [29,30].

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