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Bioreceptor multi-walled carbon nanotubes@Fe_3O_4@SiO_2-surface molecular imprinted polymer in ultrasensitive chemiluminescence biosensoring bovine hemoglobin

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Abstract

An ultrasensitive chemiluminescence (CL) biosensor with high selectivity based on bioreceptor surface molecular imprinted polymer (SMIP) which using core-shell Fe_3O_4@SiO_2-multi-walled carbon nanotubes nanostructures (Fe_3O_4@SiO_2/MWCNTs) as backbone materials for bovine hemoglobin (BHb) was proposed. The Fe_3O_4@SiO_2/MWCNTs was synthesized with a new method, and then was characterized by SEM, FTIR and XRD techniques. Adsorption ability of the Fe_3O_4@SiO_2/MWCNTs-SMIP was evaluated to be 91 mg/g and Fe_3O_4@SiO_2/MWCNTs-SMIP followed Langmuir isotherm equation and it demonstrated the excellent recognition and adsorption ability for the imprinted cites which located on the surface or near the surface of the Fe_3O_4@SiO_2/MWCNTs. Under the optimum conditions of CL, the detection range of BHb was from 5.0×10^{-10} to 7.0×10^{-7} mg/mL with the detection limit of 1.5×10^{-10} mg/mL (3δ). The proposed biosensor was successfully applied in determination of BHb in real samples with high selectivity and sensitivity, and the recoveries were excellent and varied from 92% to 106%. Finally, the possible CL mechanism of the BHb amplifying the CL signal of luminol-NaOH-H_2O_2 system was discussed.

Keyword: chemiluminescence biosensor; surface molecular imprinted polymer; bioreceptor;

multi-walled carbon nanotubes; Fe_3O_4@SiO_2; bovine hemoglobin

1. Introduction

Chemiluminescence (CL), usually coupled with chemical reaction process, was a radiation phenomenon which liberated energy by light emission. For the past few years, bioimaging, pharmaceutical and environmental analysis et al. [1] has confirmed the importance of CL technique due to its high sensitivity, no background interference and simple instrument. However, the extensive application of CL was still restricted owing to its low selectivity which could be improved by molecular imprinting [2].

In the early 1970s, Wulff et al. [3] proposed molecular imprinting technique which made the selectivity to target molecules improved greatly. A higher accuracy and lower detection limit were achieved for Wulff's contribution in the substance analyses for the preparation of polymer with specific cavities decorated with functional groups [4]. Then, the whole century witnessed the booming of molecular imprinting technique. Although selectivity to small molecules could be improved, the preparation of molecularly imprinted polymer (MIP) for biomacromolecules, such as proteins, remained a challenge. In 1987, Keyes [5] and Dabulis [6] et al. have studied bioimprinting respectively. Surface molecular imprinting technique was synthesizing a polymer attaching the biomolecule to the surface or in the proximity of the surface of the polymer, which made the mass transfer improved and the removal of the template biomolecule promoted [7]. Atom transfer radical polymerization, discovered by Kato et al. [8], was introduced in the preparation of surface molecular imprinting polymer (SMIP) bioreceptor [9].

Fe_3O_4 nanoparticles (NPs) were superior stabilizer and separation reagent used in many fields [10] because of their good biocompatibility, low toxicity, stable physical properties and easy preparation. In 2011, Gao et al. successfully prepared magnetic

SMIP on the surface of Fe_3O_4 NPs which served as the support materials to prepare biomolecule imprinted polymer by imprinting bovine serum albumin, ribonuclease A and lysozyme [11]. Si NPs, as one of the most promising one-dimensional materials, were applied in sensors, batteries, catalysts and so on for their particular properties. In 2010, He et al. synthesized SMIP over vinyl modified silica NPs via surface graft copolymerization using low monomer concentration, which was in aqueous media with lysozyme as template protein [12]. When deposited on the surface of Fe_3O_4 NPs，SiO_2 NPs possessed abundant carboxylic groups that could immobilize protein template. Fe_3O_4@SiO_2 SMIP exhibited high adsorption capacity to the biomolecular and adsorption equilibrium could be easily reached, and SMIP could be separated easily using an external magnetic field [13]. Carbon nanotubes, which were one dimensional quantum materials, had extraordinary biocompatible, mechanical and electrical properties. And then, Zhang et al. prepared MIP on the surface of MWCNTs-Fe_3O_4 composite [14].

Hemoglobin (Hb) was the main undertaker of organic life for a variety of physiological activities. It played critical roles in the transportation of oxygen and carbon dioxide and maintenance of the pH balance in the blood stream of the vascular system [15]. The structure of Hb on its transport oxygen function made respiratory function efficient. Dysregulation of the structure of Hb in its four molecular subunits could result in various kinds of hereditary diseases, such as sickle cell anemia, thalassemia and so on [16]. Thus, the analysis of Hb was of important significance. While bovine hemoglobin (BHb) shared 90% similarities with human hemoglobin [17], we could make BHb as the target protein in the analysis of Hb. During the past decades, many methods have been reported on the determination of BHb, for example, electrochemical methods [18, 19], fluorescence method [20] and piezoelectric crystal

immunosensor [21].

This work proposed an ultrasensitive chemiluminescence (CL) biosensor to BHb. The nanocomplex of Fe_3O_4 NPs, SiO_2 NPs and MWCNTs was served as backbone materials simultaneously to prepare bioreceptor SMIP for the CL biosensor for highly sensitive detection of BHb. In brief, Fe_3O_4@SiO_2 was not only served as backbone material to prepare BHb SMIP, but also as separation material to separate SMIP complex and MWCNTs was used as supporting material to bear Fe_3O_4@SiO_2 and prepare SMIP for their large specific surface area. Adsorption ability of the Fe_3O_4@SiO_2/MWCNTs-SMIP to BHb was researched, and Fe_3O_4@SiO_2/MWCNTs-SMIP followed Langmuir isotherm equation and it exhibited excellent recognition and adsorption ability to BHb owing to the imprinted cites located on the surface or near the surface of the Fe_3O_4@SiO_2/MWCNTs. Finally, based on Fe_3O_4@SiO_2/MWCNTs-SMIP bioreceptor, the proposed CL biosensor was successfully applied in detection of BHb in samples with high sensitivity and selectivity under the optimum conditions of CL.

2 Experiment

2.1 Chemicals and materials

Anhydrous tetrahydrofuran (99%), N-N methylene double acrylamide (MBA, A.R), N,N,N',N'-tetramethyl ethylenediamine (TEMED, A.R), Diethyl amino ethyl methacrylate (DMAEMA, 99%) and 3-aminopropyltrimethoxysilane (AMPS, 97%) were purchased from Aladdin Industrial Co. (China); Thionyl chloride (A.R), Tetraethyl silicate (TEOS, A.R), Ferrous sulfate(A.R), Dimethyl formamide (DMF, 99%), Acrylamide (AM, 99%), Methacrylic acid (MAA), and Ammonium persulphate (APS, AR) were supplied by Sinopharm Chemical Reagent Co. Ltd (China); MWCNTs was obtained from Beijing Dk Nano technology Co., Ltd (China);

BHb were acquired from Shanghai Reagent Co. (China). The ethanol, acetic acid, methanol, luminol and all the other chemicals unless specified were of analytical reagent grade and used without further purification.

TEOS and DMF were distilled reduced vacuum pressure and DMAEMA was purified with alkaline Al_2O_3 column. Redistilled water was used throughout the work. Phosphate buffer (PBS, pH=7.4, 0.02 mol/L) solution was used to prepare all BHb solutions which were stored in refrigerator (4°C).

2.3 Apparatus

The IFFM-E flow injection CL analyser (Xi'an Remex Electronic instrument High-Tech Ltd., China) was equipped with an automatic injection system and a detection system. PTFE tubes (0.8 mm i.d.) were used to connect all of the components in the flow system. Capillary filled with a certain amount Fe_3O_4@SiO_2/MWCNTs-SMIP bioreceptor and non-imprinted polymer (Fe_3O_4@SiO_2/MWCNTs-SNIP) bioreceptor was collected with pump by PTFE tubes and was placed in front of the CL analyser as recognition elements as shown in Fig. 1. When BHb solution ran through the capillary, BHb molecule could be absorbed by Fe_3O_4@SiO_2/MWCNTs-SMIP selectivity, a CL signal I_0 was obtained, while Fe_3O_4@SiO_2/MWCNTs-SNIP could not absorb BHb molecule, another CL signal I_1 was obtained. Then the difference $\Delta I = I_1 - I_0$ was the concentration of BHb in the linear relationship. XRD measurement was made on a D8 focus spectrometer (Brooke AXS, Germany). FT-IR spectrometer (PerkinElmer, USA) was employed to confirm the products. A FEI QUANTA FEG250 field emission scanning electron microscopy (SEM, USA) was employed to observe the morphology of the nanoparticles.

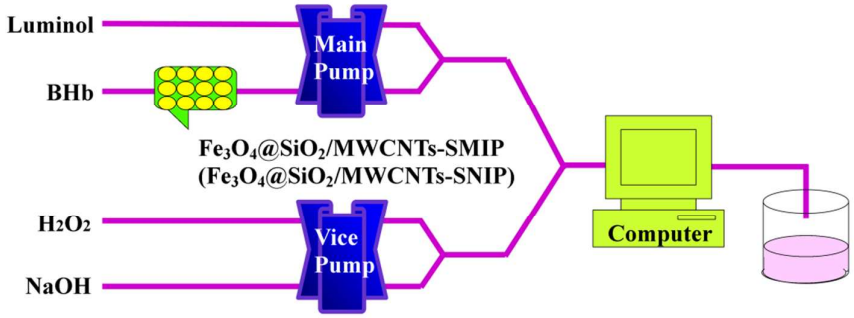

Fig.1. The mechanism of CL biosensor based on Fe_3O_4@SiO_2/MWCNTs-SMIP bioreceptor

2.3 Preparation of Fe_3O_4@SiO_2

Fe_3O_4@SiO_2 was synthesized according to a modified procedure described in the previous literatures [22] and our group [23].

$FeCl_2 \cdot 4H_2O$ (35 mg) and $FeCl_3 \cdot 6H_2O$ (50 mg) were dissolved in 80 mL of water with vigorous stirring under nitrogen protection. 10 mL of $NH_4 \cdot OH$ (28 wt. %) was added into the system drop by drop, and the reaction was maintained at 80 ◦C for 30 min. The black precipitation was separated with an external magnetic field, and washed with water and ethanol to remove the unreacted chemicals, and then dried in the vacuum at 60 °C. Subsequently, 0.3 g of as prepared Fe_3O_4 NPs were dispersed in 36 mL of ethanol and 4 mL of ultrapure water by ultrasonication for 15 min in 150 mL round bottom flask, followed by the addition of 10 mL of $NH_4 \cdot OH$ and 5 mL of TEOS. The mixtures were reacted for 12 h at room temperature by constant mechanism stirring. The products (Fe_3O_4@SiO_2) were collected by magnetic separation, washed with water and ethanol, and then dried in the vacuum at 50 °C.

2.4 Preparation of Fe_3O_4@SiO_2/MWCNTs

0.5 g Fe_3O_4@SiO_2 NPs were dispersed in 50 mL of anhydrous toluene. Then, 5 mL of AMPS was added in the solution followed by refluxing at 80 °C for 16 h. After magnetic separated, washed by water and ethanol, dried in vacuum, products (Fe_3O_4@SiO_2-NH_2) were obtained.

The carboxylated MWCNTs (MWCNTs-COOH) were prepared by refluxing the MWCNTs in a nitric acid (65-68 wt. %) at 75 °C for 11 h [24]. After cooling to room temperature, the mixture was filtered and washed with double distilled water till the pH=6.5 and finally dried at 60 °C overnight. 0.3 g MWNTs-COOH was dispersed in 25 mL thionyl chloride solution by ultrasonication (200W, 40 kHz). The mixture was further stirred vigorously and refluxed for 24 h at 50 °C. Then the product (MWCNTs-COCl) was washed by anhydrous tetrahydrofuran followed dried at 50 °C.

10 mg of MWCNTs-COCl and 20 mg Fe_3O_4@SiO_2-NH_2 were scattered in 20 mL DMF by ultrasonication which then was refluxed at 120 °C for 24 h under the protection of N_2. The obtained product (Fe_3O_4@SiO_2/MWCNTs) was then separated, washed by water and ethanol and dried in vacuum.

2.5 Preparation of Fe_3O_4@SiO_2/MWCNTs-SMIP and NIP bioreceptor

Fe_3O_4@SiO_2/MWCNTs-SMIP was synthesized according to a modified procedure described in the previous literatures [12]. The preparing process was shown in Fig. 2.

Solution A: AM (16 mg), MBA (32 mg), MAA (0.1 mL) and DMAEMA (0.1 mL) were dissolved in 25 mL of PBS solution and mixed thoroughly by ultrasonication. And then, 32 mg of BHb was dissolved to this solution by sonication.

Solution B: 120 mg Fe_3O_4@SiO_2/MWCNTs was dispersed in 15 mL ethanol and 10 mL PBS solution which was then ultrasonicated to make it well-distributed.

After adding solution B to solution A, the mixture was degassed under vacuum for 10 min and purged with nitrogen stream for another 10 min. Then, the solution was incubated for 1 h to preassemble. By adding 30 mg of APS, 0.4 mL TEMED and 15 mg ferrous sulfate to the mixture, polymerization was initiated and continued under violent stirring at 25 ◦C for 10 min.

After the reaction, the BHb-imprinted particles were collected by magnetic separation. The particles were washed with deionized water solution. And then, they were washed repeatedly with 0.5 mol/L NaCl solution to remove embedded template until no BHb in the supernatant. Subsequently, they were washed with PBS solution to remove remained NaCl solution. Finally, the Fe_3O_4@SiO_2/MWCNTs-SMIP bioreceptor was freeze-dried for further use. The Fe_3O_4@SiO_2/MWCNTs-SNIP bioreceptor were prepared and washed in the same way but without addition of BHb.

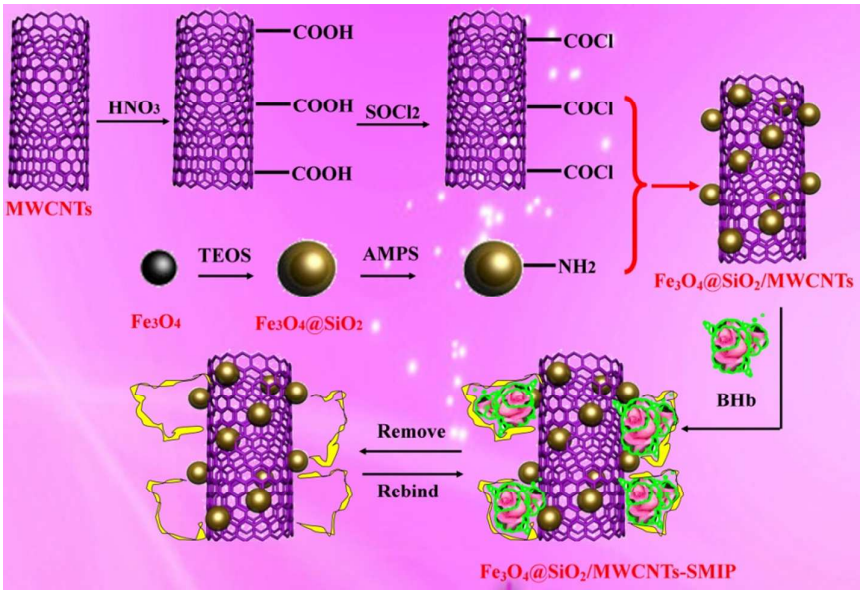

Fig.2. The preparing process of Fe_3O_4@SiO_2/MWCNTs-SMIP bioreceptor

2.6 Rebinding performance of Fe_3O_4@SiO_2/MWCNTs-SMIP and Fe_3O_4@SiO_2/ MWCNTs-SNIP

Batch rebinding tests: 55 mg Fe_3O_4@SiO_2/MWCNTs-SMIP and Fe_3O_4@SiO_2/ MWCNTs-SNIP NPs were placed into 5 mL centrifuge tubes, respectively. The protein solutions were prepared at different initial concentration: 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, 1.0 mg/mL and 1.5 mg/mL. Then, 2.0 mL of each solution was added into the tube and thoroughly mixed with the particles. The dispersion liquid was incubated at 25 ◦C for 1 h. After magnetic separation, the

concentration of the supernatant in the tube was determined by CL instrument.

Rebinding kinetics: 55 mg Fe_3O_4@SiO_2/MWCNTs-SMIP and Fe_3O_4@SiO_2/ MWCNTs-SNIP NPs were soaked in 2.0 mL 2.0 mg/mL BHb solution which then was incubated at 25 ◦C for 1 min, 5 min, 10 min, 20 min, 30 min and 40 min. After magnetic separation, the concentration of the supernatant in the tube was determined by CL instrument.

All the tests were conducted in triplicates. The amount of protein adsorbed by the particles was calculated from the following formula.

$$Q = (c_0 - c_e)\frac{V}{m}$$

Where Q (mg/g) was the mass of protein adsorbed by unit mass of dry particles, c_0 (mg/mL) and c_e (mg/mL) were the concentrations of the initial and final solution, respectively, V (mL) was the total volume of the adsorption mixture, and m (g) was the mass of the Fe_3O_4@SiO_2/MWCNTs-SMIP (Fe_3O_4@SiO_2/MWCNTs-SNIP) used.

2.7 Selectivity experiments of the biosensor

The selectivity experiments of the Fe_3O_4@SiO_2/MWCNTs-SMIP-CL biosensor was carried out with other two kinds of proteins: bovine serum albumin (BSA) and lysozyme (Lys). A certain concentration of common substances and proteins were added into BHb standard solution (5.0×10^{-8} mg/mL) to research the effects on the CL intensity.

3 Results and discussion

3.1 Characterization of Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2/MWCNTs

The surface morphology of the Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2/MWCNTs was characterized by SEM and was shown in Fig. 3, respectively. As it shown in Fig. 3 (A), the image showed that the prepared Fe_3O_4@SiO_2 NPs were well shaped beads

and well dispersed. Fig. 3 (B) showed that when reacted with MWCNTs, Fe_3O_4@SiO_2 were anchored on the surface of MWCNTs which made surface molecular imprinting more favorable by improving mass transfer. Hence, we considered that the Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2/MWCNTs were synthesized successfully.

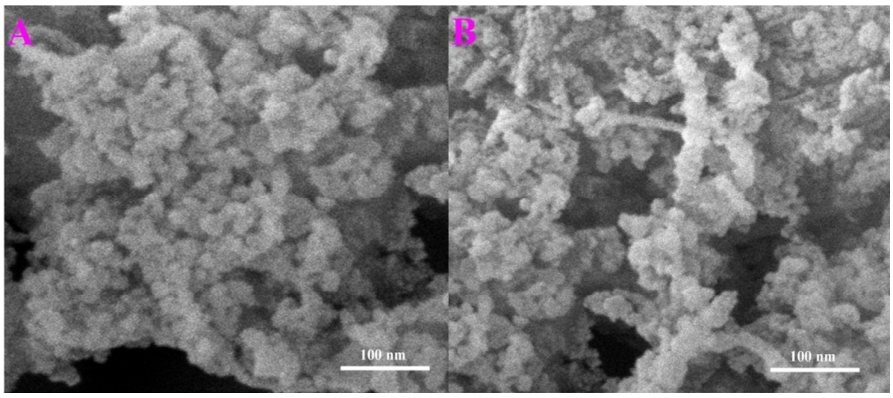

Fig.3. The SEM images of Fe_3O_4@SiO_2 (A) and Fe_3O_4@SiO_2/MWCNTs (B)

The FTIR spectra of MWCNTs-COOH, Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2/ MWCNTs were recorded within the range of 4000 - 500 cm^{-1} and were showed in Fig. 4 (A). The peak at 630 cm^{-1} was the characteristic peak of Fe_3O_4. It could be seen that the stretching vibration of Si-O bond contributed to the strong absorption at 1098 cm^{-1}. In the spectra of Fe_3O_4@SiO_2/MWCNTs, peaks at 1477 and 1674 cm^{-1} were due to the stretching vibration of benzene ring and C=O respectively, which provided abundant evidence of the satisfactory preparation of Fe_3O_4@SiO_2/MWCNTs.

In the XRD patterns of Fe_3O_4, Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2/MWCNTs shown in Fig.4 (B), the diffraction peaks at 2θ = 30.2°, 35.5°, 42.7°, 53°, 57.2° and 62.5° were the six characteristic peaks of Fe_3O_4. In the pattern of Fe_3O_4@SiO_2/MWCNTs, the broad and obviously strong diffraction peak around 2θ = 26° was corresponded to the MWCNTs [25] and amorphous form of SiO_2 [26], overlapping Fe_3O_4@SiO_2 and MWCNTs, which created a wealth proof that Fe_3O_4@SiO_2/MWCNTs was prepared

nicely.

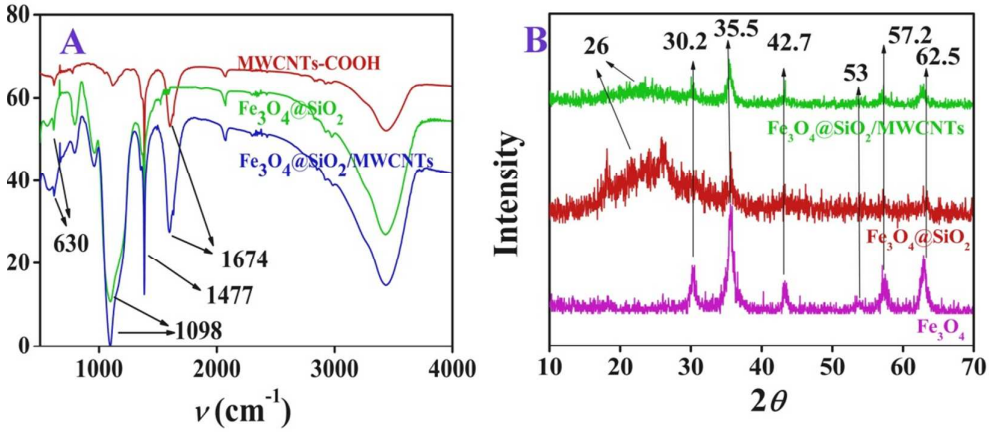

Fig.4. The FTIR spectra (A) of MWCNTs-COOH, Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2/MWCNTs. The XRD pattern (B) of Fe_3O_4, Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2/MWCNTs

3.2 Rebinding capacity and kinetics

The adsorption performance of Fe_3O_4@SiO_2/MWCNTs-SMIP and Fe_3O_4@SiO_2/MWCNTs-SNIP to BHb were performed and was shown in Fig. 5. Rebinding capacities to BHb at different initial concentrations, i.e. adsorption isotherms, were showed in Fig. 5 (A). The adsorption capacity to BHb increased with the increasing of the BHb concentration before reaching maximum 91 mg/g which was higher than that of the SNIP prepared at the same conditions. Compared to the maximum adsorption amount with Fe_3O_4@SiO_2/MIP 77.6 mg/g [27], silica-Fe_3O_4 NPs MIP 4.30 mg/g [28] and Si-NPs/CdTe/MIP 19.7 mg/g [20], it certified that Fe_3O_4@SiO_2/MWCNTs-SMIP was more advantaged to recognize BHb clearly. Theory adsorption capacity of Fe_3O_4@SiO_2/MWCNTs-SMIP was described by Langmuir isotherm shown by the inset of Fig. 5 (A). The adsorption to BHb was monolayer absorption on the surface of Fe_3O_4@SiO_2/MWCNTs-SMIP. The theory maximum adsorption capacity to BHb obtained by Langmuir isotherm was 98 mg/g which was approximate to the experimental result 91 mg/g.

The adsorption rate of Fe_3O_4@SiO_2/MWCNTs-SMIP and Fe_3O_4@SiO_2/

MWCNTs-SNIP to BHb, i.e. adsorption kinetics, was then studied and shown in Fig. 5 (B). As we could observe in the figure, both the SMIP and SNIP particles could reach the maximum adsorption within 20 min for the imprinting cavities on the surface or in the proximity of the surface of Fe_3O_4@SiO_2/MWCNTs-SMIP which could help mass transfer.

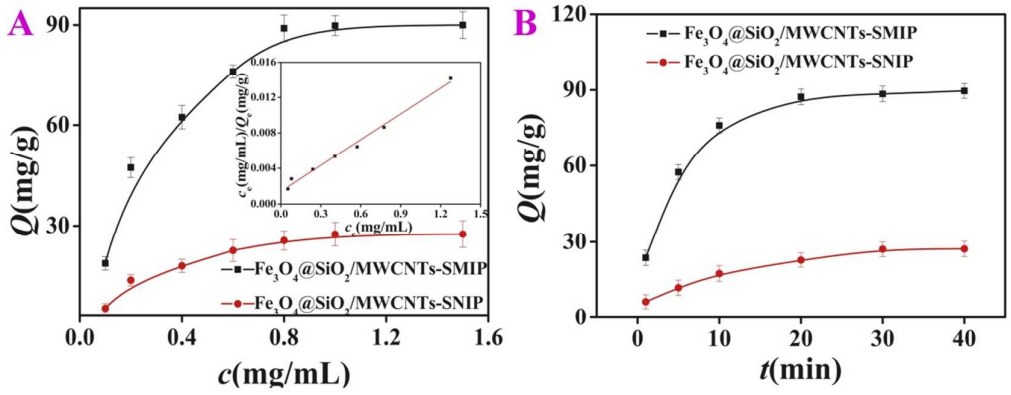

Fig.5. Adsorption isotherm curve of Fe_3O_4@SiO_2/MWCNTs-SMIP and Fe_3O_4@SiO_2/MWCNTs-SNIP (A). Adsorption kinetics curve of Fe_3O_4@SiO_2/MWCNTs-SMIP and Fe_3O_4@SiO_2/MWCNTs-SNIP (B)

3.3 Optimization of Fe_3O_4@SiO_2/MWCNTs-SMIP-CL biosensor

Peristaltic pumps speed which determined the adsorption time to BHb was an important parameter in the experiment. While main pump speed and vice pump speed reached the best: 25 r/min, the effects of concentrations of hydrogen peroxide and sodium hydroxide on the CL reaction was researched. As shown in Fig. 6 (A-C), the optimal concentration conditions were 0.03 mol/L of NaOH solution, 0.04 mol/L of H_2O_2 solution and 7.2×10^{-4} mol/L of luminol solution, respectively.

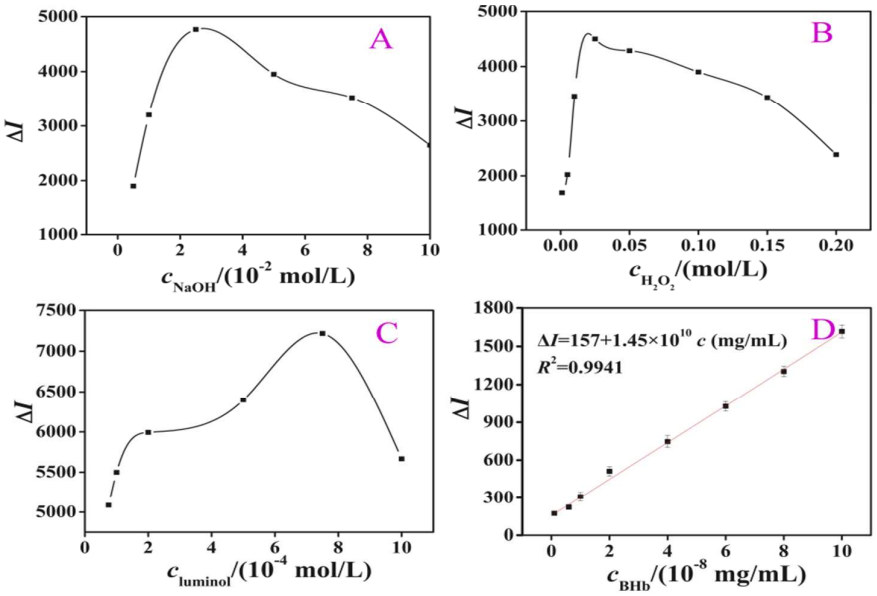

Fig.6. Optimization results: (A) Effect of NaOH concentration on CL intensity. Conditions: $c(H_2O_2)$ = 0.1 mol/L, c(luminol) = 1.0 × 10^{-5} mol/L . (B) Effect of H_2O_2 concentration on CL intensity. Conditions: c(luminol) = 1.0 × 10^{-5}mol/L, c(NaOH) = 0.03 mol/L. (C) Effect of luminol concentration on CL intensity. Conditions: $c(H_2O_2)$ = 0.04 mol/L, c(NaOH) = 0.03 mol/L. (D) The regression equation of the Fe_3O_4@SiO_2/MWCNTs-SMIP-CL biosensor

3.4 Analytical performance of the biosensor

The analytical performance of the described Fe_3O_4@SiO_2/MWCNTs-SMIP-CL biosensor was studied in the optical conditions (c(luminol) = 7.2 × 10^{-4} mol/L, $c(H_2O_2)$ = 0.04 mol/L, c(NaOH) = 0.03 mol/L). The regression equation shown in Fig. 6 (D) was described by the calibration curve ΔI = 157+1.45 × 10^{10} c (mg/mL, R^2 = 0.9941), and the detection range of BHb was from 5.0 × 10^{-10} to 7.0 × 10^{-7} mg/mL. The detection limit was 1.7 × 10^{-10} mg/mL (3δ) with relative standard deviation (RSD) 4.9% (n = 11) by determination of 5.0 × 10^{-8} mg/mL BHb. The detection range and limit was compared with traditional methods and the results were shown in Table 1. The results showed that Fe_3O_4@SiO_2/MWCNTs-SMIP-CL biosensor exhibited low detection limit, high sensitivity and selectivity to BHb conceivably.

Tab.1. Comparing results with conventional methods

Method	Linear range (mg/mL)	Detection limit (mg/mL)
Our work	5.0×10^{-10} - 7.0×10^{-7}	1.7×10^{-10}
MIPs/AuE [5]	1.0×10^{-9}-1.0×10^{-3}	
Fluorescence method [6]	1.3×10^{-3}-0.13	6.1×10^{-4}
Piezoelectric crystal immunosensor [7]	0.001-0.1	

3.5 Interference study of the biosensor

The detail information of interference study of the Fe_3O_4@SiO_2/MWCNTs-SMIP-CL biosensor was shown in Table 2. The tolerable limit of coexisted species was less than ±5%. In the CL biosensor, 150 times Na^+ and K^+ concentration (compared with BHb) interfered with the determination of BHb but when using Fe_3O_4@SiO_2/MWCNTs-SMIP biosensor, 900 times Na^+ and K^+ concentration would interfere with the determination of BHb for the imprinting cavities which were more adequate for macromolecule BHb and could recognize and absorb BHb specifically, while the interferences from BSA and Lys were serious relatively. It was shown that the BSA exhibited much more interference than Lys which was for that BSA had nearly the same size with BHb. Even so, from Table 2, it was easy to say that the application of Fe_3O_4@SiO_2/MWCNTs-SMIP in CL biosensor could eliminate or reduce the interference.

Tab.2. The tolerable ratio of interfering species to BHb

Species	Na^+, K^+	Vanillin	Lys	BSA
Fe_3O_4@SiO_2/MWCNTs-SMIP-CL	900	700	100	40
CL	150	90	35	15

3.6. Recyclability of Fe_3O_4@SiO_2/MWCNTs-SMIP bioreceptor

The recyclability of Fe_3O_4@SiO_2/MWCNTs-SMIP was an important parameter

of the biosensor, and it was evaluated by reusing Fe_3O_4@SiO_2/MWCNTs-SMIP. The BHb in used Fe_3O_4@SiO_2/MWCNTs-SMIP was extracted with 0.5 mol/L NaCl and water respectively for 7 times for better binding capability to the BHb. It could be obtained that there was 7.1% binding capacity loss within 10 times of Fe_3O_4@SiO_2/MWCNTs-SMIP to the concentration of 5.0×10^{-8} mg/mL BHb, which manifested that Fe_3O_4@SiO_2/MWCNTs-SMIP was very suitable in the determination of BHb in practical samples directly.

3.7 Application of the biosensor

As shown in Table 3, Fe_3O_4@SiO_2/MWCNTs-SMIP-CL biosensor was applied in biological samples which were made by mixing waste water containing unknown amount BHb in the optimal experimental conditions. Obviously, the recoveries from the samples were excellent and varied from 92% to 106%. These results showed that the biosensor had good accuracy to the determination of BHb. Therefore, the Fe_3O_4@SiO_2/MWCNTs-SMIP-CL biosensor used for the determination of BHb was practical.

Tab.3. Application of biosensor

Sample	c /(10^{-8} mg/mL) (n=6)	RSD%	Added(10^{-8} mg/mL)	Found(10^{-8} mg/mL) (n=6)	Recovery%
Sample 1#	1.3	3.2	5.0	6.5	104
Sample 2#	4.1	3.3	5.0	8.7	92
Sample 3#	5.5	3.7	5.0	10.8	106
Sample 4#	8.6	3.5	5.0	13.4	96

3.8 The possible CL mechanism of the biosensor

In alkaline condition, there was an overlapping broad absorption band of hemoglobin and hemin between 350 and 450 nm before oxidation in UV-vis spectra,

which would disappear when hemoglobin and hemin was oxidized. Hence, the possible CL mechanism of hemoglobin might be similar to that of hemin. In alkaline and H_2O_2 solution, by losing globin, hematin was generated. By analogy with the proposal for the porphyrin-catalyzed decomposition of peroxide into free radicals, a complexing of the peroxide with the central iron ion was formed in this reaction firstly [29]. Then, a heme-peroxy free radical was formatted. This radical could then act as the primary oxidant of luminol and increase its reactivity. Alternatively, the heme-peroxy free radical could dissociate to form a peroxy free radical which would function as the oxidant. Then, luminol was oxidized by HO· to be oxidized-state luminol which then returned to the ground-state and released photons. This could enhance the CL emission as shown in Fig.7.

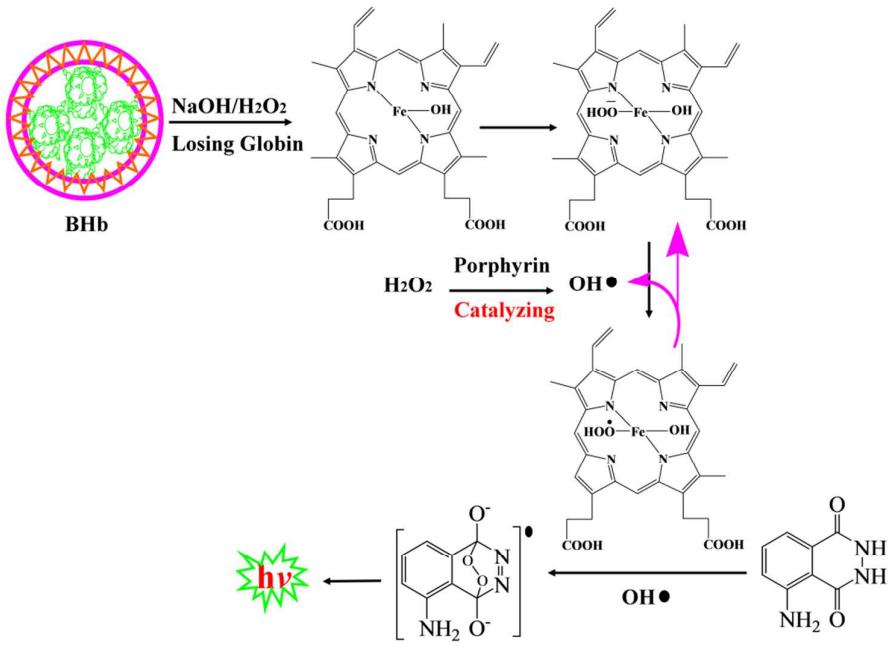

Fig.7. The possible CL mechanism of the biosensor

4 Conclusion

In this work, based on Fe_3O_4@SiO_2/MWCNTs as backbone materials to prepare SMIP bioreceptor, a CL biosensor for ultrasensitive determination of BHb was prepared. Compared with Arvand´s et al. work [30], the prepared

Fe_3O_4@SiO_2/MWCNTs with the new method was more stable on account of chemical bond. Adsorption ability of the Fe_3O_4@SiO_2/MWCNTs-SMIP was evaluated to be 91 mg/g. The Fe_3O_4@SiO_2/MWCNTs-SMIP followed Langmuir isotherm equation and exhibited excellent recognition and adsorption ability to BHb. Under the optimum conditions of CL, the Fe_3O_4@SiO_2/MWCNTs-SMIP-CL biosensor was very valuable in determining BHb in samples with high selectivity and sensitivity.

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