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A chemiluminescence sensor for determination of lysozyme using magnetic graphene oxide multi-walled carbon nanotubes surface molecularly imprinted polymers

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Abstract

In this paper, a new chemiluminescence (CL) sensor possessing high selectivity and sensitivity was established for determination of lysozyme using magnetic graphene oxide-multi-walled carbon nanotubes surface molecularly imprinted polymer (MGO-MWCNTs/SMIP). The MGO-MWCNTs/SMIP was characterized by X-ray Diffraction (XRD), Fourier Transform Infrared spectra (FT-IR) and Scanning Electron Microscopy (SEM), and the maximum adsorption capacity of MGO-MWCNTs/SMIP to lysozyme was researched to be 140 mg/g. The MGO-MWCNTs/SMIP was fixed into glass tube and was connected to the chemiluminescent analyzer. Then MGO-MWCNTs/SMIP-flow injection chemiluminescence (MGO-MWCNTs/SMIP-CL) sensor based on luminol-NaOH-H_2O_2 CL system for the determination of lysozyme was established. The proposed sensor with high selectivity and sensitivity responded linearly to the concentration of lysozyme over the range 5.04×10^{-9} - 4.27×10^{-7} g/mL and the detection limit was 1.90×10^{-9} g/mL (3δ). The recoveries were ranging from 98% to 111% when determining lysozyme in eggs, and the result was satisfactory. The advantageous properties of sensor hold the potential to be applied in protein analysis, analogizing to biological analysis.

Key words: lysozyme; magnetic graphene-oxide; surface molecular imprinting; flow injection chemiluminescence; multi-walled carbon nanotubes

1. Introduction

As a basic enzyme which can hydrolyze mucopolysaccharide of the pathogens [1-2], lysozyme widely exists in most living body [3]. Lysozyme plays an important role in immune regulation aspects of human and is closely related to human health [4] with good antibacterial,

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anti-inflammatory and anti-virus etc [5]. For now, lysozyme is mainly used in biochemical research and has multiple applications in clinical, such as the treatment of herpes and warts [6-9]. So far, some methods such as ion exchange method [10] and resonance light scattering method [11-12] etc have been used for determination of lysozyme. However, these methods suffer from poor selectivity, low sensitivity, poor anti-interference ability and expensive equipment. Therefore, development of a novel method for lysozyme detection is very important. Recent years, surface molecules imprinting (SMIP) technique with good selectivity has received much more attention. Molecularly imprinting refers to the technology that prepares the polymer with selective recognition ability to target molecular. Deng [13] had synthesized a copper ion selective membrane by surface-modified molecular imprinting. And the adsorption selectivity of the ion imprinted membrane was increased. Zayats [14] had obtained maltose imprinting in hydrogels by surface molecules imprinting technique and the recognition of protein was tuned at the molecular level.

Flow Injection (FI) is a rapid online analytical technique [15]. Chemiluminescence (CL) technology has the advantages of high sensitivity and wide linear range etc [16]. The FI-CL was established with combination of these two methods. This method has the merits of high sensitivity and wide linear range [17], and it is widely used in biology [18], pharmacy [19], environmental science [20] and many other areas. Because of poor selectivity, FI-CL could not be directly used in analyzing complex samples.

The combination of SMIP with high specific recognition and FI-CL with high-sensitivity in the establishing the novel sensor has great significance. Interference from coexisting substances and complex sample matrices was eliminated. Thereby, the anti-jamming capability of surface molecules imprinting chemiluminescence (SMIP-CL) analysis was substantially strengthened.

In this work, using MGO-MWCNTs as the supporting material, the lysozyme MGO-MWCNTs/SMIP was obtained. Due to the presence of MWCNTs, specific surface area of GO were increased obviously and site of action were increased accordingly. And the agglomeration of GO was reduced by the existence of MWCNTs. With the advantages of easy separation property of magnetic Fe_3O_4 nanoparticles, high adsorption ability of GO-MWCNTs and excellent specificity recognition of SMIP, the overall performance of MGO-MWCNTs/SMIP was increased dramatically. And then, the adsorption properties of the polymers were studied. Then, the MGO-MWCNTs/SMIP-CL sensor was constructed with high selectivity and high sensitivity and the

FI-CL analytical sensor for determination of lysozyme was established.

2. Materials and methods

2.1 Reagents

All the chemicals were of analytical reagent grade unless otherwise stated. Double-distilled water was used throughout this work. Lysozyme, acrylamide, methacrylic acid were purchased from Sinopharm Chemical Reagent Company (China). MWCNTs were purchased from Beijing Daojin Technology Company (China). Graphite was purchased from Tianjin Hongyan Chemical Reagent (China). 3-(methacryloyloxy) propyl trimethoxysilane (MPS), N,N,-methylene-bis-acrylamide, Diethylaminoethyl methacrylate (DMAEMA), Ethylene glycol dimethacrylate (EGDMA), N,N,N N,-tetramethylethylenediamine were purchased from Aladdin Reagent Company (China).

2.2 Apparatus

The IFFM-E flow injection CL analyzer was purchased from Xi'an Remex Electronic Instrument High-Tech Ltd. The schematic of Luminol-NaOH-H_2O_2 CL system used in this study was shown in Fig. 1. The FI-CL analyzer was equipped with an automatic injection system and a detector. All of the components were connected with the flow system using polytetrafluoroethy-lene tube (0.8 mm i.d.). The CL signal was analyzed with a personal computer.

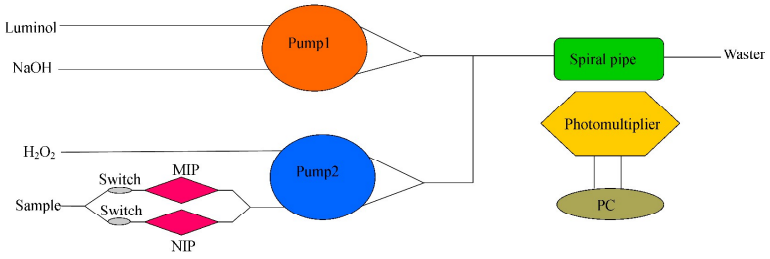


Fig. 1 The schematic of flow injection chemiluminescence system.

2.3 Preparation of MGO-MWCNTs/SMIP of lysozyme

GO was prepared according to the reported procedure from natural graphite powder by a modified hummers method [21]. Firstly, 1.0 g graphite powder was added into 500 mL three-necked flask. Then, 200 mL mixed acid solution (180 mL H_2SO_4 + 20 mL HNO_3 solution) was added into the flask, cooled by immersion in an ice bath and stirred for 0.5 h. Subsequently, 6.0 g $KMnO_4$ was slowly added and the reaction was carried out for 2 h while the temperature was kept to be 90 °C. In this process, the condensed city water was continuously for 12 h. In the hood, the ice bath was then removed and H_2O_2 was slowly dropwised added until the reaction was completed. The solution was

kept stirring until no gas generation. The final product was then centrifuged, washed twice with 30 mL 0.2 mol /L HCl solution and several times with 95% ethanol. Finally, the product was dried in vacuum and brown GO was obtained.

GO-MWCNTs composites were obtained by using a previously reported procedure [22] with modification. 1.0 g MWCNTs was dissolved in 70 mL HNO_3 solution (65~68 wt.%). The suspension was stirred and refluxed for 11 h, and the temperature was kept below 75 °C. The product was washed with distilled water until pH=6.5. The product was dried under a vacuum environment for 10 h at 80 °C. 0.3 g GO was stripped in 80 mL ethanol-water mixture (*V:V*=1:1) less than 3 h by sonication. 0.4 g acidified MWCNTs was added to the alcohol-water solution at room temperature. Then, the mixed solution was sonicated for 30 min. The solution was transferred to a 200 mL teflon-lined stainless steel autoclave and maintained at 200 °C for 6 h. Thereafter, the product was cooled to room temperature. The black cylindrical product was achieved and was washed seven times with ethanol. Finally, he product of GO-MWCNTs was gained after drying at 60 °C under vacuum.

MGO-MWCNTs composites were synthesized by using a previously reported procedure [23]. 50 mg GO-MWCNTs was dissolved in 30 mL ultrapure water and ultrasonic dispersing 0.5 h. 50 mg $FeCl_3$ and 35 mg $FeCl_2$ were added into this solution and supplemented with 20 mL ultrapure water. Under the protection of N_2, the solution was stirred and heated at 90 °C. $NH_3 \cdot H_2O$ (28%) was added into the solution until pH =9.0 and the solution was heated with stirring for 0.5 h. the solution was separated with a magnet after heating and cooling to room temperature, washed twice with ethanol and dried in a vacuum oven.

In this experiment, 0.3 g magnetic nanoparticles were added into a mixed solution (2 mL MPS and 20 mL anhydrous toluene). Under the protection of N_2, the solution was refluxed for 12 h. Finally, the product was collected by an external magnetic field, washed with ultrapure water and obtained the product of silane modification.

Subsequently, 16 mg N,N-methylene bisacrylamide ,32.6 mg acrylamide, 0.1 mL methacrylate, 0.1 mL DMAEMA, 0.1 mL EGDMA, 32 mg lysozyme and 25 mL phosphate buffer solution (PBS, pH=7.47, *c*: 0.01 mol/L) were added into 250 mL iodine flask and sonicated. Then, 120 mg MGO-MWCNTs was dissolved in 15 mL ethanol and 5 mL PBS solution by ultrasonication. Then, two solutions were mixed quickly and shocked for 1 h at 25 °C, then 30 mg $(NH_4)_2S_2O_8$, 0.4 mL

N,N,N,N,-tetramethylethylenediamine and 15 mg $FeSO_4$ were added into this solution. Subsequently, solution was shocked under nitrogen protection for 2 h at 25 ℃. The product was washed twice with distilled water. In order to remove the unreacted monomers and the template molecule, the product was washed by NaCl (0.5mol/L) solution under ultrasonication. In the next step, to remove excess NaCl, the product was washed twice with distilled water and dried at 60 ℃.

The MGO-MWCNTs/SNIP was obtained using the same way without the addition of lysozyme. The preparation process of MGO-MWCNTs/SMIP was shown in Fig. 2.

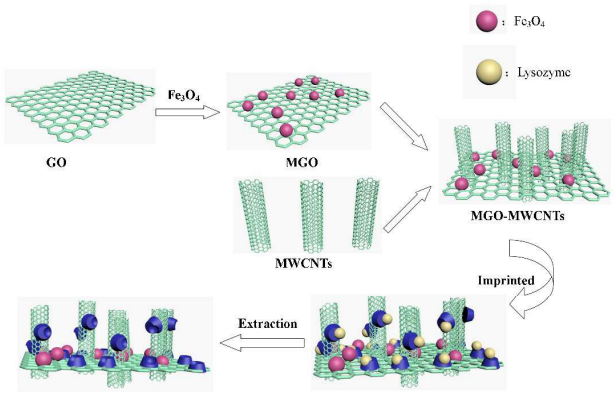


Fig. 2 The preparation process of MGO-MWCNTs/SMIP.

2.4 Adsorption tests of MGO-MWCNTs/SMIP and MGO-MWCNTs/SNIP

MGO-MWCNTs/SMIP and MGO-MWCNTs/SNIP were added into the lysozyme solution for adsorption under the same conditions. The same volume of lysozyme solution was taken for test at different times to get the adsorption capacity. And a series of lysozyme standard solutions with different concentrations were prepared, and the same amounts of SMIP and SNIP were added into the solutions for adsorption of the lysozyme.

2.5 Procedure for determination of lysozyme

The schematic for the CL sensor was shown in Fig. 1 and specific experimental steps were shown as follows:

（1） Enrichment of lysozyme. Deputy pump (pump 1) stopped working. The injection valve was in the sampling position and the main pump (pump 2) transferred lysozyme solution flowing through the column of MGO-MWCNTs/SMIP for 60 s. The lysozyme was absorbed by MGO-MWCNTs/SMIP.

（2） Removing of residual impurities. Pump 1 stopped working, the injection valve was at the

sampling location and the Pump 2 transferred water to wash other substances except for lysozyme.

（3）Determination of lysozyme. Pump 2 and Pump 1 were started and the injector valve was in the injection position. H_2O_2 and luminol solution flowed together in the column of MGO-MWCNTs/SMIP for 50 s and reacted with lysozyme which generated a CL signal.

（4）Cleaning of the MGO-MWCNTs/SMIP column. Pump 1 stopped working and the injection valve was in the sampling position. The main pump transferred water through the column of MGO-MWCNTs/SMIP for 70 s. The residue was washed away from the column of MGO-MWCNTs/SMIP for the test again.

3. Results and discussion

3.1 Characterization

FT-IR spectra was recorded by fourier transform infrared spectroscopy (Perkin–Elmer,USA) with KBr pellet and was used to investigate the chemical groups on the surface of MGO-MWCNTs, MGO-MWCNTs/SMIP and MGO-MWCNTs/SNIP, and the results were shown in Fig. 3.

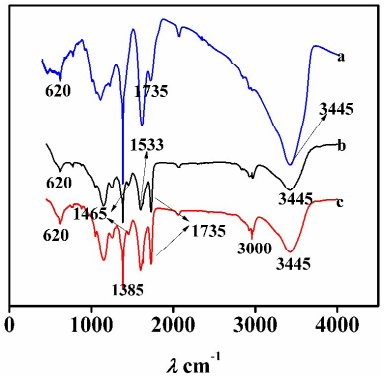


Fig. 3 The FT-IR spectrum of MGO-MWCNTs (a), MGO-MWCNTs/SMIP (b) and MGO-MWCNTs/SNIP (c).

In the spectrum of MGO-MWCNTs, the peak at 3445 cm^{-1} was the telescopic symmetrical characteristic peak of -OH. 1735 cm^{-1} was the characteristic peak of carboxyl. The peak at 620 cm^{-1} was the characteristic peak of Fe_3O_4 which gives evidence to the successful preparation of the MGO-MWCNTs. In the spectrum of MGO-MWCNTs/SMIP, 1385 cm^{-1}(the characteristics of $–CH_3$), 1465 cm^{-1} (the characteristics of $–CH_2$) and 1533 cm^{-1} (the characteristics of –NH–) was able to justify the preparation of the SMIP. Compared to MGO-MWCNTs/SNIP, the characteristic peak at 3000 cm^{-1} has an obvious displacement effected by hydrogen bonding. The results proved that hydrogen bonding existed in synthesized SMIP.

X-ray diffraction (XRD) measurements were employed to investigate the phase and structures

of GO, MWCNTs, MGO-MWCNTs and Fe_3O_4. As shown in Fig. 4a, the GO show a characteristic peak at 2θ=10.9° shown the GO was synthesized successfully [24]. Fig. 4b showed the typical XRD patterns of the MWCNTs and the sharp peak was located at 2θ=26°. The peaks in Fig. 4d at 2θ values of 30.2°, 35.5°, 43.2°, 53.6°, 57.1° and 62.7° were consistent with the standard XRD data of Fe_3O_4. All characteristic peaks of GO, MWCNTs and Fe_3O_4 were included in Fig. 4c which gives evidences of the successful preparation of the MGO-MWCNTs.

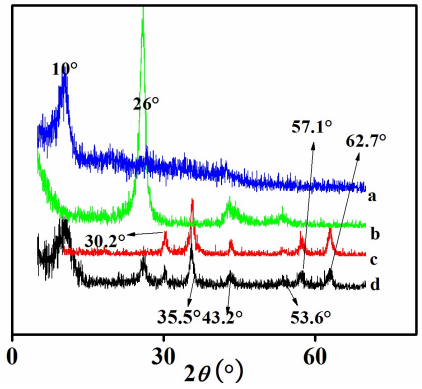


Fig. 4 XRD patterns of GO (a), MWCNTs (b), Fe_3O_4 (c) and MGO-MWCNTs (d).

Fig. 5 showed the SEM images of the obtained GO, GO-MWCNTs and MGO-MWCNTs. As shown in Fig. 5, the GO (a) presents the sheet-like structure, smooth surface, and wrinkled edge. In addition, GO and MWCNTs were uniformly intertwined as shown in Fig. 5b. It proved that GO-MWCNTs were synthesized successfully, and Fe3O4 had coated on the GO-MWCNTs surface as shown in Fig. 5c [25]. The MGO-MWCNTs/SMIP was shown in Fig. 5d and MGO-MWCNTs/SNIP was shown in Fig. 5e are nano-particles, and roughness of the surface was different. The MGO-MWCNTs/SMIP, it contains cavities of lysozyme, the surface of particles was rough, and MGO-MWCNTs/SNIP was smooth without imprinted cavities. The MGO-MWCNTs/SMIP was prepared with imprinted cavities.

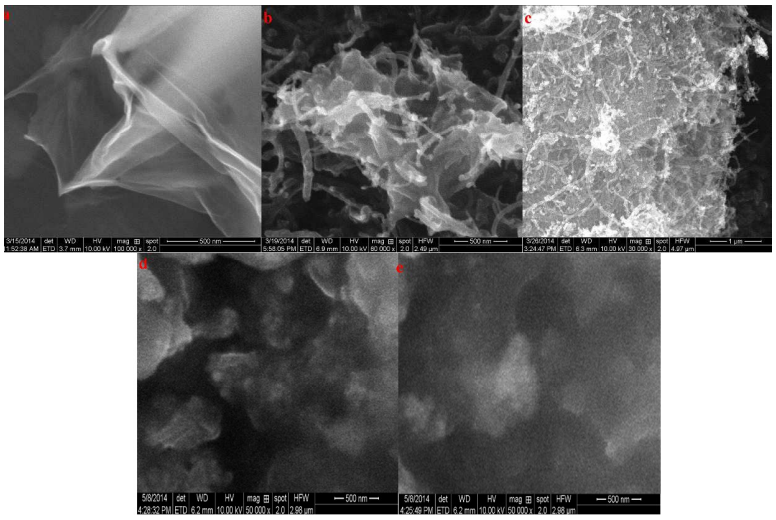


Fig. 5 SEM images of the obtained GO (a), GO-MWCNTs (b), MGO-MWCNTs (c), MGO-MWCNTs/SMIP(d) and MGO-MWCNTs/SMIP(e).

The BET surface area of MGO-MWCNTs/SMIP and MGO-MWCNTs/SMIP estimated from Barrett–Joyner–Halenda (BJH) analysis of the isotherms were determined. The surface area of MGO-MWCNTs/SNIP was 24.214 m^2/g and the surface area of MGO-MWCNTs/SMIP was 57.295 m^2/g. Obviously, the surface area of MGO-MWCNTs/SMIP was larger than that of MGO-MWCNTs/SNIP.

3.2 Adsorption study of MGO-MWCNTs/SMIP and MGO-MWCNTs/SNIP

The adsorption property of MGO-MWCNTs/SMIP to lysozyme was shown in Fig. 6a. As it can be seen from the adsorption kinetics of MGO-MWCNTs/SMIP, lysozyme bounded to the polymers quickly at the beginning of adsorption. The adsorption amount of MGO-MWCNTs/SMIP increased and reached the maximum adsorption (140 mg/g) rapidly. At the beginning of adsorption, the hole of the SMIP could quickly capture the lysozyme molecule. When the most binding sites on the surface were occupied, the adsorption rate of SMIP to lysozyme decreased gradually due to the large steric hindrance. Similarly, the SNIP molecules adsorbed amount increased rapidly at the beginning stages of adsorption, but the adsorption capacity of SNIP was less than SMIP. The result can be explained that there were no specific recognition cavities to lysozyme in SNIP molecules.

Fig. 6b showed that the adsorption capacity of MGO-MWCNTs/SMIP molecules and MGO-MWCNTs/SNIP molecule, and the adsorption capacity increased with the increasing concentration of lysozyme. And when the concentration of lysozyme reached a certain concentration,

the adsorption capacity of the MGO-MWCNTs/SMIP and MGO-MWCNTs/SNIP would not increase. However, the adsorption capacity of MGO-MWCNTs/SNIP (95 mg/g) is much lower than the adsorption amount of MGO-MWCNTs/SMIP (140 mg/g). This is due to there is no imprinted cavities in MGO-MWCNTs/SNIP, only MGO and MWCNTs were involved in the adsorption of lysozyme. But, not only MGO and MWCNTs were involved in the adsorption of lysozyme in MGO-MWCNTs/SMIP, but also the MGO-MWCNTs/SMIP molecule has a specific recognition cavity for lysozyme, which makes the adsorption capacity of SMIP further increased. Therefore, the differences between MGO-MWCNTs/SMIP and MGO-MWCNTs/SNIP in adsorption appeared. In addition, a large hydrophobic groove (the active site of lysozyme) is present on the surface of lysozyme. And there are a large number of amino and carboxyl groups on the surface of lysozyme. So lysozyme can interact with many small molecules. Therefore, the nonspecific adsorption of lysozyme is serious and the adsorption capacity of MGO-MWCNTs/SMIP is only 50% higher than that of MGO-MWCNTs/SNIP.

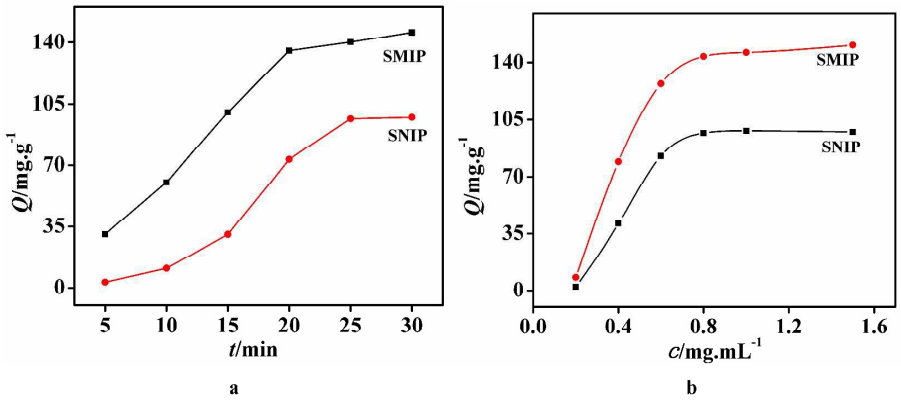


Fig. 6 The adsorption capacities of MGO-MWCNTs/SMIP and MGO-MWCNTs/SNIP.

3.3 Optimization of MGO-MWCNTs/SMIP-CL sensor

The diagram of FI-CL was shown in Fig. 1. The pump 2 speed, the pump 1 speed and concentrations of the luminescent reagents were optimized.

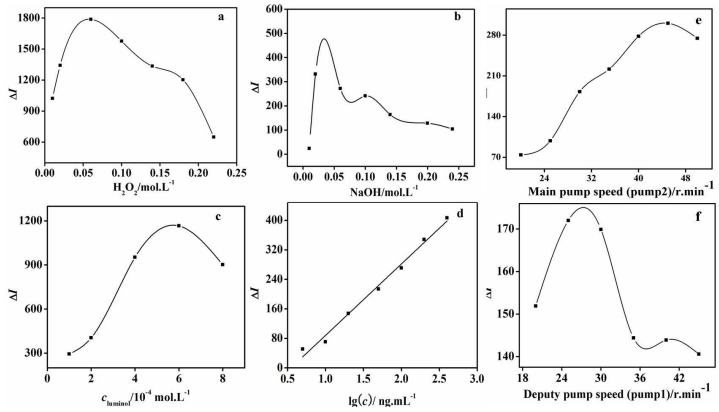


Fig. 7 Optimization results. (a) Effect of H_2O_2 concentration on CL intensity. (b) Effect of NaOH concentration on CL intensity. (c) Effect of luminol concentration on CL intensity. (e) Effect of main pump (pump 2) speed on CL intensity. (f) Effect of deputy pump (pump 1) speed on CL intensity.

The experimental results were shown in Fig. 7. FI-CL signal was greatly affected by the concentration of H_2O_2 (Fig. 7a). With the H_2O_2 concentration in the range from 0.02 to 0.06 mol/L, the CL intensity reached maximum at the 0.05 mol/L concentration of H_2O_2. Thus, the optimum concentration of H_2O_2 was 0.05 mol/L.

The effect of concentration of NaOH solution on CL intensity was investigated in Fig. 7b. When NaOH concentration was in the scope of 0.01-0.24 mol/L, the CL intensity increased with the concentration of NaOH up to 0.03 mol/L. However, when the concentration of NaOH solution over 0.03 mol/L, the CL intensity decreased. Thence, 0.03 mol/L was the ideal choice as the concentration of NaOH solution.

The influence of concentration of luminol solution was examined over the range of 1.0×10^{-4} - 8.0×10^{-4} mol/L, the CL intensity reached maximum when the concentration of luminol was 6.0×10^{-4} mol/L as shown in Fig. 7c.

The effect of main pump (pump 2) speed on luminous intensity was explored in Fig. 7e. As we can observed, the optimal pump speed of pump 2 was 45 $r.min^{-1}$.

The deputy pump (pump1) speed was studied on CL intensity. As shown in Fig. 7f. The optimal pump speed of pump 1 was 25 $r.min^{-1}$.

3.4 Analytical performance of sensor

Under the optimum conditions, the linearly response range of the sensor for lysozyme was obtained as shown in Fig. 7d. The linear equation expressed as ΔI=-1.14×10^{2}+1.99×10^{2}lg$c$ (c was the lysozyme concentration) and the correlation coefficient was 0.9950. The detection limit was 1.90×10^{-9} g/mL and the sensor responded linearly to the lysozyme over the range 5.04×10^{-9} -

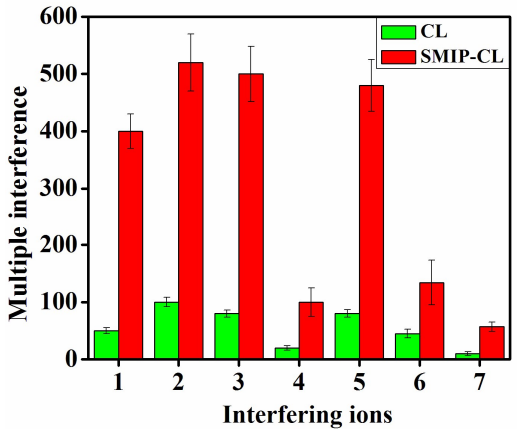
244 4.27×10^{-7} g/mL. Thence, it is proved that the method has low detection limit.

245 Then the sensor was placed in a vacuum oven. Two weeks later, the sensor was used to detect
246 lysozyme and the sensor performance did not change significantly. The RSD was within the
247 acceptable range. Therefore, the sensor was in good stability.

248 **3.5 Interferences study**

249 Under the exploring conditions (luminol: 6.0×10^{-4} mol/L, NaOH: 0.03 mol/L, H_2O_2: 0.05 mol/L,
250 pump 2 speed: 45 r/min, the pump 1 speed: 25 r/min). Coexisting substances was added into
251 lysozyme solution (3.0×10^{-8} mol/L^{-1}) to investigate the effect on CL intensity. variety of substances
252 were added into lysozyme solution to examine the effects for determination the lysozyme. The
253 tolerable fold of interfering substances in sample with MGO-MWCNTs/SMIP and
254 MGO-MWCNTs/SNIP column was compared when relative error was less than±5% and the
255 tolerance times are shown in Fig. 8.

256 These result showed that after MGO-MWCNTs/SMIP was used to sensor, the detection of
257 lysozyme was not affected by 400 times the concentration of Fe^{3+}, 520 times the concentration of
258 Na^{+}, 500 times the concentration of citric acid, 100 times the concentration of cytochrome C, 480
259 times the concentration of lactate, 130 times the concentration of bovine serum albumin and 50 times
260 the concentration of bovine hemoglobin. The anti-jamming capability of sensor was increased
261 obviously. Thus, the sensor could be used to lysozyme analysis and the selectivity has been increased
262 dramatically.



263

264 Fig. 8 Interference study. 1: Fe^{3+}; 2. Na^{+}; 3: citric acid; 4: cytochrome C; 5: lactate; 6: bovine serum albumin; 7: bovine hemoglobin.

265 **3.6 Application of MGO-MWCNTs/SMIP-CL sensor**

266 The sensor was used to detect lysozyme in eggs. Egg samples need to be processed before: the

egg was cracked and egg white was diluted by PBS (pH=7.47, *c*: 0.01 mol/L). The solution was bathed at 77 ℃ for 10min and was centrifuged in order to obtain the supernatant. Then, the supernatant was diluted tenfold. And according to the test method, the spiked recovery experiment was carried out.

The result was shown in Table 1. The recoveries varied from 98% to 111%. As a result, the CL sensor used for the determination of lysozyme was practical.

Table 1 Determination results of samples (*n*=6).

Samples	Content(ng/mL）	Added(ng/mL）	Found(ng/mL）	Recovery(%)	RSD(%)
1#	200	100	311	111	3.4
2#	100	100	198	98	3.7
3#	50	100	156	106	4.1

3.7 Possible mechanism of the reaction

The structure of lysozyme consists of α- helix, β- fold, β- corner and random coil. And the lysozyme was a globular protein contained 129 tactic amino acid residues with the active center in the cleft between the two domains on the molecule surface[26].Six tryptophan residues are located at the binding site of lysozyme and the 62th and 108th of the tryptophan residues are burned the main groups.

In view of the widespread use of H_2O_2 in protein modification, the mechanism of H_2O_2 oxidizing certain amino acids in lysozyme is not clear up to now. However, Song [27] has proved that lysozyme can enhance the chemiluminescence signal and the possible mechanism of the reaction was proposed. Therefore, it may be concluded that the enhancement mechanism is presented below:

$$\text{tryptophan (in lysozyme)} + H_2O_2 + OH^- \rightarrow \text{intermediate radical(in lysozyme)} \quad (1)$$

$$\text{intermediate radical(in lysozyme)} + \text{luminol} \rightarrow \text{oxidation products} + h\nu \quad (2)$$

Conclusions

In this work, the MGO-MWCNTs/SMIP which exhibited high selectivity to lysozyme was synthesized. The MGO-MWCNTs/SMIP was characterized by SEM, XRD and FT-IR. The adsorption properties of the polymer were studied. Then, a new CL method for the determination of lysozyme based on SMIP was achieved. Luminol-NaOH-H_2O_2 CL system was selected and the optimum condition for CL was explored. The proposed method responded linearly to the concentration of lysozyme over the range was 5.04×10^{-9} - 4.27×10^{-7} g/mL. The detection limit was

293 1.90×10^{-9} g/mL (3δ) which reflected the sensor was satisfactory. The advantageous properties of
294 sensor hold the potential to be applied in protein analysis, analogizing to biological analysis.

295 **Acknowledgment**

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