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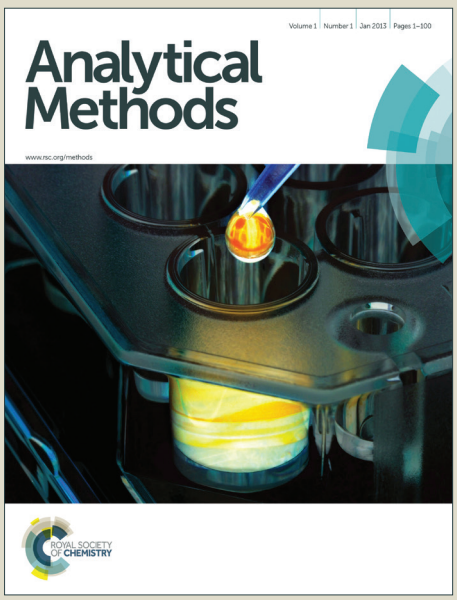

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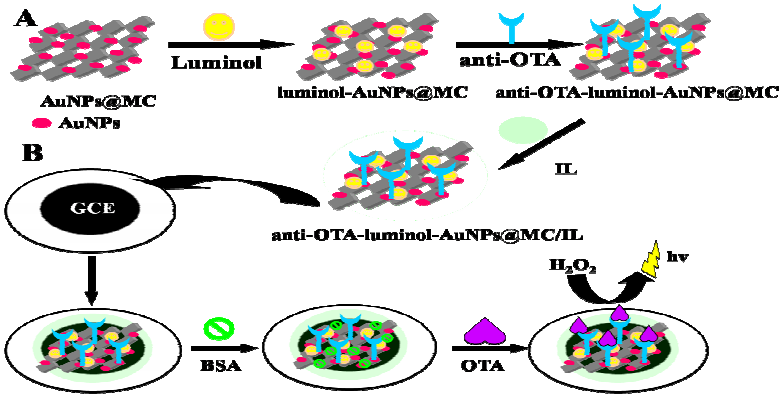
Graphical Abstract



Ultrasensitive electrochemiluminescence immunosensor for detection of ochratoxin A based on gold nanoparticles hybridized mesoporous carbon

Ultrasensitive electrochemiluminescence immunosensor for detection of ochratoxin A based on gold nanoparticles hybridized mesoporous carbon

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ABSTRACT

In this study, an ultrasensitive electrochemiluminescence (ECL) immunosensor was fabricated for rapid determination of ochratoxin A (OTA) based on gold nanoparticles hybridized mesoporous carbon (AuNPs@MC). The ECL immunosensor was constructed by modifying anti-OTA-luminol-AuNPs@MC ionic liquid (IL) dispersion on glassy carbon electrodes. AuNPs@MC presented remarkable performance of signal enhancement and IL was adopted to increase the conductivity of the ECL immunosensor. The voltage was applied by single-step cycle pulse method，which limited the continuous consumption of luminol and increased the stability of the ECL immunosensor. The proposed ECL immunosensor is simple, rapid, sensitive, specific, stable and reliable. Under optimum conditions, a wide detection range of 1 pg/mL to 50 ng/mL with a detection limit of 0.3 pg/mL was achieved. The proposed ECL immunosensor exhibited great promise in food analysis field.

Keywords: electrochemiluminescence immunosensor, ochratoxin A, single-step cycle pulse, ionic liquid, gold nanoparticles hybridized mesoporous carbon.

1. Introduction

Ochratoxin A (OTA) belongs to the group of toxic compounds produced as secondary metabolites by several aspergillus and penicillium fungal species. OTA is teratogen, mutagen, hepatotoxic and nephrotoxic agent. It is immunosuppressive to different animal species. The International Agency for Research on Cancer has classified OTA in the group 2B as a renal carcinogenic to animals and possibly to human [1, 2]. Ochratoxin-producing fungi can contaminate several crops, beverages and fruits [3-6], therefore it poses a serious risk for human and animals' health. For this reason, regulations about maximum admissible levels of OTA in food and feed have been set worldwide to protect the health of consumers [7-9].

Monitoring the presence of OTA in food and feed would be advisable to reduce risks of human and animals' health. Available analytical methods for detection of OTA including high-performance liquid chromatography, immunoaffinity columns liquid-liquid extraction and liquid chromatography have been widely used [10-16]. Nevertheless, these methods typically require skilled operators, extensive sample pretreatment and expensive equipment. Recently, Electrochemiluminescence (ECL) immunosensor has attracted intensive and extensive research interests due to its important applications in real samples analysis with the promising advantages such as simplicity,

rapidity, high sensitivity, reproducibility and low background [17-19]. Moreover, luminol-H_2O_2 ECL system with typical of stable signal has aroused some concern due to its briefness, low oxidation potential, inexpensive reagent consumption and high emission yield [20].

Gold nanoparticles (Au NPs) are such materials which could accelerate electron transfer [21]. Au NPs have the virtues of unique physical properties, good biocompatibility and ease of synthesis and functionalization [22]. Biomolecules such as proteins could be easily immobilized on Au NPs *via* electrostatic interaction and physical adsorption. Moreover, more biomolecules can be immobilized on Au NPs due to the high surface-to-volume ratio. In this work, Au NPs are assembled on the surface of mesoporous carbon (MC). MC is a kind of typical mesoporous materials with high surface area, large pore volume and good stability [23], which greatly facilitate its application in numerous fields such as hydrogen storage [24] and electric double layer capacitors [25]. The MC allows the high loading of Au NPs and antibodies, and enhances the ECL signal intensity.

In recent years, ionic liquid (IL) is of immense interest as it has physicochemical properties which different from conventional molecular solvents [26]. IL is composed purely of ions in the liquid state with no solvent. It generally has good ionic conductivity, wide electrochemical windows, electrochemically stability and the ability to dissolve a broad

variety of materials [27, 28]. It is confirmed that IL is a good biocompatible material and it has been used as the modifier for the fabrication of biosensors [29].

In this paper, an ultrasensitive label-free ECL immunosensor was developed for the first time based on gold nanoparticles hybridized MC (AuNPs@MC) for the determination of OTA. IL was applied to provide a favorable microenvironment and promote electron transport to enhance the sensitivity of the immunosensor. The obtained immunosensor exhibited good response for detection of OTA and showed great potential application in real sample analysis.

2. Experimental

2.1. Materials

OTA and anti-OTA were purchased from Huaan Magnech Bio-Tech Co., Ltd. (Beijing, China). Luminol (1mmol/L) and bovine serum albumin (BSA, 96-99%) were obtained from Sigma-Aldrich (Beijing, China). 1-butyl-3-methyl-immidazolium tetrafluoroborate ($BMIM{\cdot}PF_4$, >99%) was obtained from the Lanzhou Institute of Chemical Physics (Lanzhou, China). Mesoporous carbon (MC) was obtained from XFNANO Co., Ltd. (Nanjing, China). $HAuCl_4{\cdot}H_2O$ was purchased from Shanghai reagent (Shanghai, China). Carbonate buffer solutions (CBS) were prepared using 1/15 mol/L Na_2CO_3, 1/15 mol/L

$NaHCO_3$. Phosphate buffered saline (PBS) was prepared by using 1/15 mol/L KH_2PO_3, 1/15 mol/L Na_2HPO_3. Ferricyanide solutions ($Fe(CN)_6^{3-/4-}$, 5.0 mmol/L, pH 7.4) were obtained by dissolving potassium ferricyanide and potassium ferrocyanide with PBS (pH 7.4). All other chemicals were of analytical grade and were used without further purification. All solutions were stored at 4 ˚C before usage. Ultrapure water (resistivity of 18.25 MΩ·cm) was used through the experiments.

2.2. Apparatus

Scanning electron microscope (SEM) images were obtained using a field emission SEM (Zeiss, Germany). UV-vis absorbance spectra were examined with a Lambda 35 UV-vis Spectrophotometer (PerkinElmer, American). The ECL measurements were performed with a model MPI-F flowinjection chemiluminescence detector (Remax, China) and electrochemical measurements were carried out on CHI 760D electrochemical workstation (Chenhua, China). Electrochemical impedance spectroscopy (EIS) measurements were performed with IM6e Electrochemical Interface (Zahner, Germany).

2.3. Preparation of AuNPs@MC

AuNPs@MC were prepared by *in-situ* reduction. For the preparation of AuNPs@MC, 20 mg MC were ultrasonicated for 30 min to ensure

sufficient dispersion. Then, 2 mL $HAuCl_4$ (1%) was added into the solution. After adsorption at room temperature under continuous stirring for 12 h, the resulting mixture was centrifugated at 9000 rpm for 20 min to remove the excess $HAuCl_4$. Subsequently, the products were dispersed in water, and hydrochloric acid was used to adjust pH (pH ≤ 2). Then, 1 mL sodium borohydride (50 mmol/L) were dropwise added into the above mixture under continuous stirring for 30 min. At last, the as-prepared AuNPs@MC were collected by centrifugation and washed several times with ultrapure water. The obtained AuNPs@MC were redispersed in 10 mL ultrapure water.

2.4. Preparation of luminol-AuNPs@MC

Briefly, 2 mL AuNPs@MC (2 mg/mL) and 2 mL luminol were mixed and continuously shaking for 12 h at room temperature in the dark. Luminol were attached to AuNPs@MC by the chemical bonding of amino and Au NPs [30]. Afterwards, excess luminol was removed by centrifugation (9000 rpm, 10 min). The obtained solid materials were redispersed in 2 mL PBS (pH 7.4) and stored at 4 ˚C.

2.5. Synthesis of anti-OTA-luminol-AuNPs@MC/IL

Fig.1A shows the whole synthetic processes of anti-OTA-luminol-AuNPs@MC/IL. Luminol-AuNPs@MC was added into a PBS solution containing anti-OTA (1 mg/mL; pH=7.4; V=0.1 mL)

and incubated under shaking for 24 h at 4 ˚C. The products were collected by centrifugation. Subsequently, the prepared anti-OTA-luminol-AuNPs@MC was dispersed in IL and stored at 4 ˚C until use.

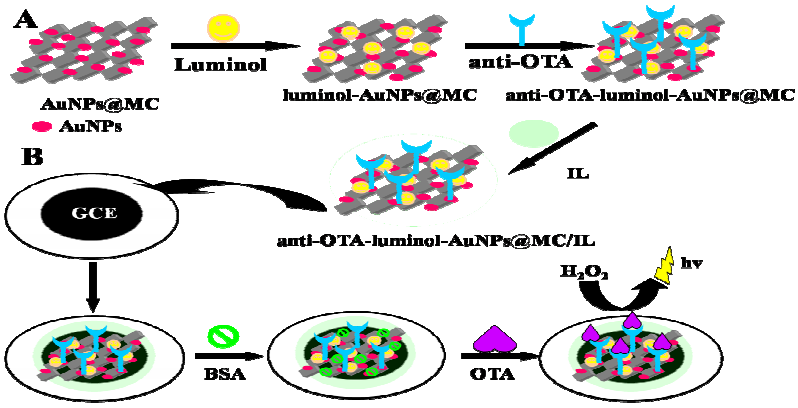

Fig. 1. (A) Fabrication process of anti-OTA-luminol-AuNPs@MC/IL and (B) Schematic diagram of the fabrication of the immunosensor.

2.6. Fabrication of the ECL immunosensor

Fig. 1B shows the schematic illustration of the fabrication processes of the ECL immunosensor. The glassy carbon electrode (GCE, 4 mm in diameter) was polished with 1.0, 0.3, and 0.05 μm alumina slurry successively to obtain a mirror-like surface, followed by rinsing with ultrapure water. Then, 6 μL of anti-OTA-luminol-AuNPs@MC/IL (2 mg/mL) was dropped on the surface of GCE. Subsequently, 3 μL of BSA solution (1%) was dropped on the anti-OTA-luminol-AuNPs@MC/IL film to block the non-specific binding sites. Next, the resulting electrode was washed with buffer solution (pH 7.4). At last, the electrode was

incubated with OTA at different concentrations. After washing, the prepared electrode was stored at 4 ˚C until use.

2.7. Measurement procedure

The ECL detection was performed at room temperature in an ECL detector cell with 10 mL CBS (pH 10.6, 1/15 mol/L) containing 2.5 mmol/L hydrogen peroxide (H_2O_2). A three-electrode system consisted of an Ag/AgCl (saturated KCl) electrode as the reference electrode, a platinum wire electrode as the auxiliary electrode and a modified GCE as the working electrode. The single-step cycle pulse was applied to obtain ECL signal. The pulse potential, pulse time, initial potential and pulse period were set at 0.8 V, 0.05 s, -0.35 V and 5 s. A voltage of 300 V was supplied to the photomultiplier for luminescence intensity determination. In this study, the ECL intensity decreased with the increasing concentration of OTA, which may be ascribed to the captured OTA on the modified electrodereduced the contact of luminol and H_2O_2 and inhibite the electron transfer.

3. Results and discussion

3.1. Characterization of AuNPs@MC and anti-OTA-luminol-AuNPs@MC

The morphology and microstructure of AuNPs@MC were characterized by SEM (Fig 2A). The image presents that MC was

uniformly covered by Au NPs (the bright points in the picture). This fact confirmed the successful assembly of Au NPs on MC. The inset was the image of size distribution of Au NPs. The mean size of Au NPs is about 24 nm. The Au NPs in this size with specific surface area can immobilize large amounts of antibodies.

To further verify the successful synthesis of anti-OTA-luminol-AuNPs@MC, the UV-vis absorption spectra were investigated (Fig. 2B). For the absorption spectra of luminol (curve a), anti-OTA (curve b), Au NPs (curve c) and anti-OTA-luminol-AuNPs@MC (curve d), the absorption peak at 300 nm and 360 nm could be ascribed to the characteristic peak of luminol (curve a). The absorption peak at about 280 nm in curve b and 520 nm in curve c are attributed to the characteristic absorption of anti-OTA and Au NPs respectively. All the absorption peaks appeared in curve c, suggesting the successful synthesis of anti-OTA-luminol-AuNPs@MC.

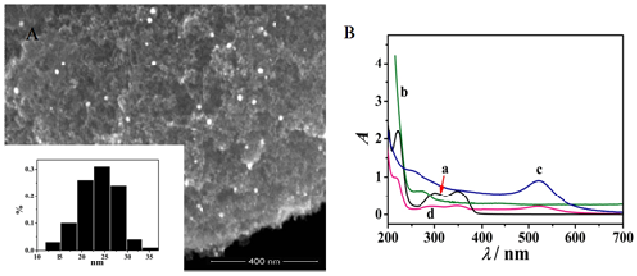

Fig. 2 SEM (A) image of Au@MC (inset was the image of size distribution of Au NPs) and UV-vis (B) absorption spectra of luminol (a), anti-OTA (b), Au NPs (c) and anti-AFB_1-luminol-Ag@MC (d).

3.2 Characterization of the immunosensor fabrication

To monitor interfacial feature of the sensor, EIS was utilized to follow the immunosensor fabrication process and probe the feature of the surface modified electrode (Fig. 3A). In the EIS, the semicircle diameter at higher frequencies corresponds to the electron-transfer resistance, and the linear part at lower frequencies corresponds to the diffusion process. The electron transfer resistance increased with the increase of semicircle diameter. The bare GCE exhibited very small diameter of the semicircle (curve a), which was characteristic of a diffusion process, indicating that there were no materials to stand in the way of electron transport. After the formation of the luminol-AuNPs@MC/IL nanocomposites film, the EIS was a straight line basically (curve b) because of the favorably high electrical transport properties of Au NPs and MC. When anti-OTA was bonded onto the electrode surface, the electron transfer resistance increased greatly (curve c) because of its poor conductivity. The subsequent immobilization of BSA resulted in a further increased resistance (curve d). Finally, OTA was conjugated on the former layer, and the electron transfer resistance was further increased indicating that an insulating layer prevented the diffusion of redox probe to the electrode surface. The results were consistent with the fact that the electrode was modified as expected. With the step-wise modification of electrode, the ECL signal decreased (Fig. 3B), which further proved that the electrode

was modified successfully.

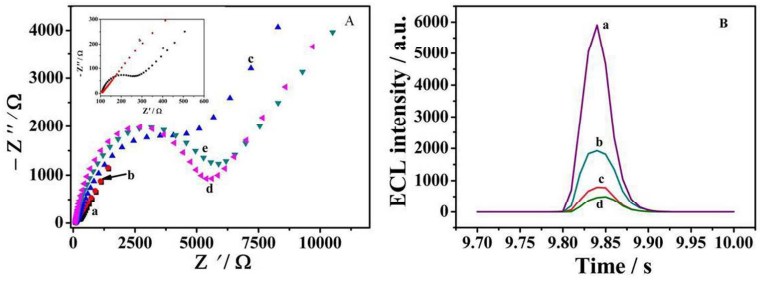

Fig. 3 (A) EIS of the electrode at different stages (inset is the larger version of a and b): (a) bare GCE, (b) luminol-AuNPs@MC/IL/GCE, (c) anti-OTA-luminol-AuNPs@MC/IL/GCE, (d) BSA/anti-OTA-luminol-AuNPs@MC/IL/GCE and (e) OTA/BSA/anti-OTA-luminol-AuNPs@MC/IL/GCE measured in ferricyanide solutions ($Fe(CN)_6^{3-/4-}$, 5.0 mmol/L, pH 7.4); (B) ECL of electrode at different stages: (a) luminol -AuNPs@MC/IL/GCE, (b) anti-OTA-luminol-AuNPs@MC/IL/GCE, (c) BSA/anti-OTA-luminol-AuNPs@MC/IL/GCE and (d) OTA/BSA/ anti-OTA-luminol-AuNPs@MC/IL/GCE measured in CBS (pH 10.6) containing 2.5 mM H_2O_2.

3.3 Optimization of detection conditions

To obtain the maximal ECL intensity, the effects of pH, H_2O_2 concentration, pulse potential, initial potential, pulse time and pulse period on the ECL intensity were investigated. As shown in Fig. S1 (supplemental materials), the optimal conditions were as follows: 10.6 pH CBS, 2.5 mmol/L H_2O_2, 0.8 V pulse potential, 0.05 s pulse time, -0.35 V

initial potential and 5 s pulse period.

3.4. Performance of the proposed immunosensor

3.4.1. Calibration curve

Under the above optimum conditions, we investigated the quantitative range of the proposed ECL immunosensor for the detection of OTA. The ECL intensity decreased with the increase of OTA concentrations by the label-free structure. The ECL immunosensor presented a good linearity with the concentration of OTA in the range of 1 pg/mL to 50 ng/mL with a detection limit of 0.3 pg/mL (S/N = 3) (Fig. 4). The linear relationship can be represented as I = 1310.27 - 408.22 log c with the correlation coefficient of r = 0.9986. We compared the linear range and detection limit of the proposed method with the other reported methods (Table S1). It can be observed that the proposed ECL immunosensor displayed wider linear range and lower detection limit. Although the electrochemical aptasensor [32] developed by Lin et al. had lower detection limit than ours, a label-free ECL immunosensor was more simple and faster due to its simple structure. The other techniques in table require skilled operators, extensive sample pretreatment and expensive equipments. ECL has a unique advantage for the detection of OTA due to its low cost, rapidity, low background, simple instrumentation, high sensitivity, and applicability to a wide range of analytes.

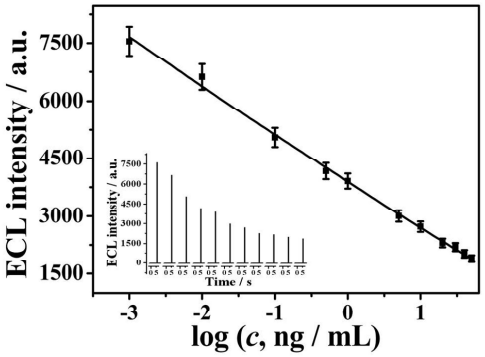

Fig. 4 Calibration curve of the immunsensor to different concentration of OTA. Error bar = RSD, n = 3.

3.4.2 Selectivity, stability and reproducibility of the ECL immunosensor

From the practical point, selectivity is an important criterion. In order to monitor the selectivity of the immunosensor, the interferences of ochratoxin B (OTB), ochratoxin C (OTC), aflatoxin B_1 (AFB_1), aflatoxin M (AFM) were investigated for the determination of OTA. As shown in Fig. 5A, only the target OTA induced an obvious variation of the ECL intensity. The variations of the ECL intensity induced by interferences could be ignored. Therefore, the selectivity of the immunosensor is good enough and the immunosensor is suitable for real samples analysis.

The operational stability of the proposed ECL immunosensor was examined by consecutively giving potential to the immunosensor 20 times for the detection of OTA (0.1 ng/mL). An up-close look at the results of the detection was obtained (Fig. 5B), and the ECL intensity

didn't show any remarkable changes.

The reproducibility of the proposed immunosensor was also examined by five prepared sensors for the detection of OTA (0.1 ng/mL). The relative deviations (RSD) were lower than 2.3%, indicating that the reproducibility of the proposed immunosensor was acceptable.

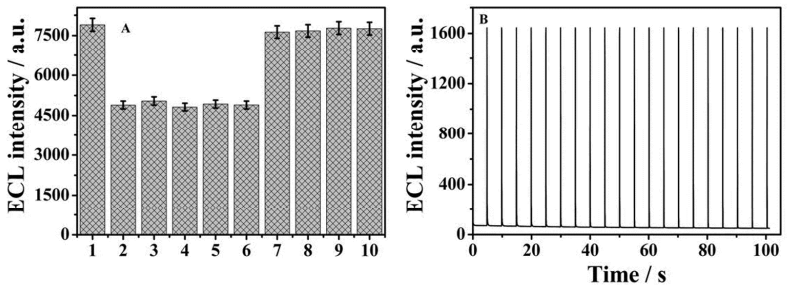

Fig. 5 (A) A comparison of ECL responses of OTA with variousinterfering species. (1) 0 ng/mL OTA; (2) 0.1 ng/mL OTA; (3) 0.1 ng/mL OTA + 10 ng/mL AFB_1; (4) 0.1 ng/mL OTA + 10 ng/mL AFM; (5) 0.1 ng/mL OTA + 10 ng/mL OTB; (6) 0.1 ng/mL OTA + 10 ng/mL OTC; (7) 0.1 ng/mL AFB_1; (8) 0.1 ng/mL AFM; (9) 0.1 ng/mL OTB; (10) 0.1 ng/mL OTC. (B) ECL intensity of anti-OTA-Luminol-AuNPs@MC/IL/ GCE incubated with OTA 0.1 ng/mL in CBS (pH=10.6) containing 2.5 mM H_2O_2 continuous scanning for 20 cycles. Error bar = RSD, n = 3.

3.4.3 Application

The applicability of the proposed method in real samples was investigated. A series of samples were prepared by adding OTA of different standard concentration to orange juice samples (standard

addition method). The results were summarized in Table 1 demonstrated acceptable recoveries (between 97.4 %-102.8 %) and RSD (less than 1%), indicating that the developed method could be applied to the determination of OTA in real products.

Table 1: The recovery of OTA in real samples using the proposed ECL immunosensor measured in 10 mL CBS (pH 10.6) containing 2.5 mM H_2O_2.

Sample number	Added amounts (ng/mL)	Measured amounts (ng/mL)	RSD (%)	Recovery (%)
1	5.00	4.87	0.47	97.4
2	10.00	9.93	0.61	99.3
3	20.00	20.56	0.79	102.8
Measured amounts = mean value (n = 5)				

4. Conclusions

A novel label-free ECL immunosensor was developed successfully for sensitive detection of OTA based on AuNPs@MC. The immunosensor exhibited outstanding stability and sensitivity. Its virtues were attributed to the following main advantages: Firstly, the characters of MC realize a large loading amount of Au NPs. Au NPs not only had excellent catalytic performance to catalyze the luminescence of luminol and H_2O_2, but also could facilitate electron transfer. Secondly, IL had good electrical and ionic conductivity to promote the electron transfer and increase the ECL

signal. It had electrochemical stability, and enhanced the stability of the immunosensor. Lastly, the single-step cycle pulse method limited the continuous consumption of luminol by cyclic voltammetry and enhanced the stability. Moreover, the immunosensor exhibited good reproducibility and specificity. It provided a promising method for OTA detection in real samples analysis.

Acknowledgements

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