Talanta 84 (2011) 204–211

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A monoclonal antibody-based immunosensor for detection of Sudan I using electrochemical impedance spectroscopy

Fei Xiao [a], Ningdan Zhang [a], Hongjie Gu [b], Min Qian [b], Jing Bai [a], Wen Zhang [a,*], Litong Jin [a,**]

[a] Department of Chemistry, East China Normal University, Shanghai 200062, PR China
[b] School of Life Science, East China Normal University, Shanghai 200062, PR China

ARTICLE INFO

Article history:
Received 27 September 2010
Received in revised form
24 December 2010
Accepted 6 January 2011
Available online 13 January 2011

Keywords:
Monoclonal antibody
Sudan I
Immunosensor
Electrochemical impedance spectroscopy

ABSTRACT

Sudan I monoclonal antibodies (Mabs) were prepared by hybridoma technique and firstly used to develop a Sudan I immunosensor by immobilizing the Mabs on a gold electrode. o-Mercaptobenzoic acid (MBA) was covalently conjugated on the gold electrode to form a self-assembled monolayer (SAM). The immobilization of Sudan I Mabs to the SAM was carried out through a stable acyl amino ester intermediate generated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydrosuccinimide (NHS), which could condense antibodies reproducibly and densely on the SAM. The changes of the electrode behavior after each assembly step were investigated by cyclic voltammetric (CV) technique. The Sudan I concentration was measured through the increase of impedance values in the corresponding specific binding of Sudan I and Sudan I antibody. A linear relationship between the increased electron-transfer resistance (Ret) and the logarithmic value of Sudan I concentrations was found in the range of 0.05–50 ng mL^{-1} with the detection limit of 0.03 ng mL^{-1}. Using hot chili as a model sample, acceptable recovery of 96.5–107.3% was obtained. The results were validated by conventional HPLC method with good correlation. The proposed method was proven to be a feasible quantitative method for Sudan I analysis with the properties of stability, highly sensitivity and selectivity.

1. Introduction

Sudan azo dyes, include Sudan I–IV (Fig. 1), are synthetic chemical colorants and traditionally used in industrial products [1–3]. In the past few years, Sudan dyes, especially, Sudan I is also found used as an additive in foods, such as hot chili powder [3], relishes, chutneys, seasonings, sauces, and ready meals [4] because of their bright and vivid red color [5]. However, Sudan I was recognized as a potential carcinogen to both human beings and animals which was demonstrated through laboratory experiments and thus was classified as a category 3 carcinogen by the International Agency for Research on Cancer (IARC) [6–9]. Therefore, the use of Sudan I as food additive is forbidden all over the world. To this end, it is of great importance to develop a sensitive, rapid and reliable method for the determination of Sudan I in foodstuffs.

The usually proposed method for the determination of Sudan I in foodstuffs was high performance liquid chromatography (HPLC) coupled with different detectors, such as UV [1,10–12], MS [13–15], and chemiluminescence (CL) [16]. Other analytical procedures, including isotope dilution combined with MS [3], solid-phase extraction [17], and flow injection CL assay [18], have also been reported. Recently, a few electrochemical methods [9,19–21] based on electroactive groups (–N=N– and –OH) of Sudan I have also been successfully applied in the detection of Sudan I. However when Sudan I is mixed just in traces in food stuffs, developing more sensitive, selective and convenient methods for the determination of Sudan I is of great importance and interest.

Due to the highly sensitive and selective nature of the recognition between antigen (Ag) and antibody (Ab), immunoassays are very useful in widespread applications such as medical detection, processing quality control, and environmental monitoring [22]. The methods used in immunoassays involve radio-immunoassay, enzyme-linked immunosorbent assay (ELISA) [23], electrochemistry [24], chemiluminescence [25], piezoelectricity [26], surface plasmon resonance [27] and so on. Among these techniques, ELISA and electrochemical immunoassay have received much attention for its high sensitivity, specificity and low cost. Recently, several ELISA methods have been developed for the detection of Sudan I in food samples [28–32]. However to our knowledge, there is no electrochemical immunoassay available for the determination of Sudan I. As most antibodies and antigens are electrochemically inert, the technique of electrochemical impedance spectroscopy (EIS) is developed to provide a direct determination of immuno species by measuring the change of impedance. In addition to its convenience, EIS provides a nondestructive means for the char-

* Corresponding author.
** Corresponding author. Tel.: +86 21 62232627; fax: +86 21 62232627.
E-mail address: ltjin@chem.ecnu.edu.cn (L. Jin).

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doi:10.1016/j.talanta.2011.01.001

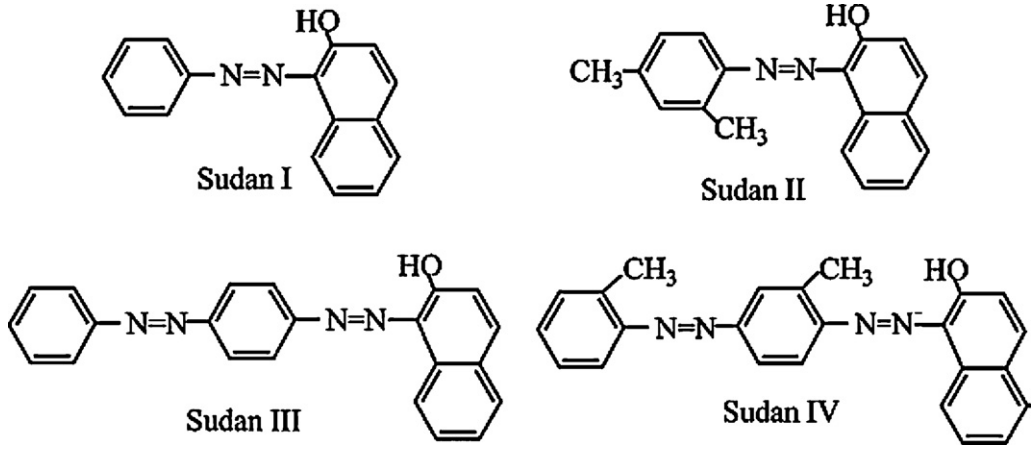

Fig. 1. Chemical structures of Sudan I–IV.

acterization of the electrical properties in biological interfaces [33,34].

In this paper, Sudan I Mabs were prepared and an electrochemical impedance immunosensor for the detection of Sudan I was developed by immobilizing Sudan I Mabs on an Au electrode. MBA was modified on Au surface to form a SAM. The immobilization of antibodies on the SAM was carried out through a stable acyl amino ester intermediate generated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and Nhydrosuccinimide (NHS). Co-addition of EDC and NHS can facilitate the formation of a suitable intermediate to condense antibodies on the SAMs and enhance the stability and sensitivity of the developed immunosensor [35,36]. The Sudan I concentration was measured through the increase of electron-transfer resistance (ΔRet) values in the corresponding specific binding of Sudan I and Sudan I Mabs. Under optimal conditions, the linear relationship between the ΔRet and Sudan I concentration was obtained in the range of 0.05–50 ng mL^{-1} with a limit of detection (LOD) of 0.03 ng mL^{-1} (3σ), which showed that the fabricated immunosensor exhibited good sensitivity. The results demonstrated that the developed immunosensor supplied a convenient, low-cost, and sensitive method for Sudan I determination. Moreover, the established method could provide an approach for the design of EIS immunosensors for analysis of other toxicant in foodstuffs in the future.

2. Experimental

2.1. Reagents, materials and buffers

2.1.1. Reagents and materials

Carboxyl derivative of Sudan I (CSD I) was from Chinaway Bio. Ltd. (Guangzhou, China). Sudan I, II, III, IV, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydrosuccinimide (NHS), bovine serum albumin (BSA), ovalbumin (OVA), goat anti-mouse IgG-HRP, complete and incomplete Freund's adjuvant, polyethylene glycol (PEG) 4000 were purchased from Sigma Chemicals (St. Louis, MO, USA). HAT (hypoxanthine/aminopterin/thymidine) and fetal calf serum (FCS) were from GIBCO BRL (NY, USA). Non-Fat Powdered Milk was purchased from BIO Basic Inc. (NY, USA). Balb/c mice were from Shanghai Laboratory Animal Center of Chinese Academy of Science (Shanghai, China). RPMI 1640 was purchased from Invitrogen (CAL, USA), o-mercaptobenzoic acid (MBA), dicyclohexylcarbodiimide (DCC), N,N-dimethylformide(DMF), methanol (HPLC grade), Tween-20, 30% hydrogen peroxide (H_2O_2) and 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All the reagents were used without further purification and the water was double distilled.

2.1.2. Buffers

(1) Coating buffer: 0.05 mol L^{-1} carbonate buffer, pH 9.6; (2) coating antigen stock solution: 1 mg mL^{-1} of coating antigen prepared with coating buffer; (3) assay buffer: 0.01 mol L^{-1} phosphate-buffered solution (PBS) pH 7.4, containing 145 mmol L^{-1} NaCl; (4) washing buffer (PBST): assay buffer with 0.1% (v/v) of Tween-20; (5) blocking solution: 5% of non-fat powdered milk in washing buffer; (6) acetate buffer: 100 mmol L^{-1} sodium acetate acid buffer, pH 5.7; (7) substrate solution (TMB + H_2O_2): 200 μL of 10 mg mL^{-1} TMB dissolved in DMF, 20 μL of 5% H_2O_2 and 1 mL of acetate buffer were added to 20 mL of pure water; (8) stop solution: sulfuric acid (5%); (9) Sudan I standard solutions were prepared by diluting the stock solution (1 mg mL^{-1}, by dissolving Sudan I red powder in DMF) with methanol:water (5:95, v/v); (10) PBS used in EIS was consisted of 0.2 mol L^{-1} sodium phosphate dibasic and potassium dihydrogen phosphate with appropriate addition of 1.0 mol L^{-1} sodium hydroxide to adjust the pH 7.0.

2.2. Apparatus

All the cyclic voltammetry (CV) and electrochemical impendence spectroscopy measurements were carried out using CHI 660 Electrochemical system (CHI, USA), which employed a three-electrode cell with the saturated calomel electrode (SCE) as the reference electrode, a platinum sheet electrode as counter electrode and the immunosensor as working electrode. Raman spectra were recorded by RamTracer-200-WF-I laser Raman spectroscopy (OptoTrace Technologies Inc., Suzhou, China). HPLC analysis was carried out on LC10AT HPLC system (Shimadzu, Japan) with a UV detector. ELISA was carried out on 96-well polystyrene microplates (COSTAR, USA). The microplate reader was EXL800 from BIOTEK (Winooski, VT, USA).

2.3. Production of monoclonal antibodies

2.3.1. Synthesis of immunogen and coating antigen of Sudan I

140 mg CSD I was dissolved in 3 mL DMF, then 103.2 mg DCC and 75 mg NHS was added into the solution with a vigorous stirring at room temperature overnight. Then the mixture was centrifuged and the supernatant was collected and added into 5 mL 0.1 M PBS buffer (pH 7.0), which contained 340 mg BSA and 0.5 mL DMF. After stirring at 4 °C for 4 h, the mixture was centrifuged and the supernatant was transferred to dialyse against normal saline for 3 days.

Finally, the CSD I-BSA solution was divided into vials and stored in −20 °C.

The preparation of CSD I-OVA conjugate was the same as that of CSD I-BSA conjugate, except that OVA was used instead of BSA. The CSD I-BSA conjugate was used as immunogens for antibody preparation, and the CSD I-OVA conjugate was used as coating antigen.

2.3.2. Immunization of mice and preparation of monoclonal antibodies

Four female Balb/c mice, 6 weeks age, were injected at 2 weeks intervals at multiple sites on the back and abdomen with 50 μg of immunogen in 0.1 mL of sterilized PBS which was emulsified with an equal volume of Freund's complete adjuvant (3 times). The first injection was given with complete Freund's adjuvant and last two with incomplete Freund's adjuvant. Ten days after the second and third injection, serum was collected from the tail of each mouse and titers of antibodies were determined by an indirect ELISA. Two weeks after the third injection, the mice with the highest titers to Sudan I received another booster injection without adjuvant at caudal vein.

Three days after the final injection, spleen cells were removed and fused with myeloma cells (SP2/0) [37] at a ratio of 10:1 (spleen cells: myeloma) using 50% poly-ethylene glycol (PEG) 4000 and then incubated in HAT culture. Ten days after fusion, the cells were selected with HT medium and divided in a 96-well culture plate. When the cell clones occupied 25–50% of the bottom surface of the well, the cell suspensions were then diluted with RPMI 1640 medium containing 10% FCS. Hybridoma cells in ELISA-positive wells were cloned by limiting dilution. The growing-well, strong positive clones were chosen to expand in vitro and retain a small part for freezing in liquid nitrogen.

Several healthy Balb/c mice, which had been injected atoleine for peritoneal cavity priming, were then inoculated hybridoma cells. After 7–10 days, the ascites were removed from abdomen and purified by caprylic ammonium sulfate precipitation. Finally, the ascites were lyophilized and stored at −20 °C until use.

2.4. Development of ELISA method

Indirect ELISA method was performed according to the procedure of Xu et al. [31]. (1) The microplates were coated with coating conjugate CSD I-OVA (100 μL well^{-1}) and incubated at 4 °C overnight. (2) Plates were washed with the washing buffer three times and blocked with 300 μL well^{-1} of block buffer, then plates were incubated at 37 °C for 1 h. (3) 100 μL of antibody with or without competitors was added to each well. After incubation for 1 h at 37 °C, the unbound compounds were washed away. (4) 100 μL of goat anti-mouse IgG-HRP was then added to each well and incubated for 1 h at 37 °C. (5) After washing three times, 100 μL of substrate solution was then added into each well and incubated for 20 min at 37 °C. (6) The enzymatic reaction was stopped by the stopping solution (50 μL well^{-1}) and then absorbance at 450 nm was measured.

2.5. Monoclonal antibody titer and sensitivity

The titer of the antibody was determined by indirect ELISA. The sensitivity to free Sudan I was determined using the indirect competitive ELISA. To prepare a standard curve, 100 μL of Sudan I standard solution (0, 0.1, 0.3, 0.9, 2.7 and 8.1 ng mL^{-1}) was added in the indirect competitive ELISA. The signals obtained in the presence of various Sudan I concentrations and without competitor (maximal signal) were referred to as B and B_0, respectively. The inhibition ratio was obtained by dividing B with B_0 (B/B_0). A linear standard curve was prepared by plotting log (Sudan I concentration) versus the inhibition ratio.

2.6. Fabrication of the immunosensor

A gold electrode (Φ = 3 mm) was firstly polished carefully with alumina slurries (1, 0.3 and 0.05 μm) and washed ultrasonically with double distilled water. After the electrode was cleaned with 0.1 M NaOH 20 min, 0.1 M HCl 5 min and Piranha solution (a 1:3 mixture of 30% H_2O_2/concentrated H_2SO_4) 5 min in turn, it was rinsed with ethanol and double distilled water successively and dried in nitrogen.

The pretreated electrode was immersed in an ethanol solution of 1.0% MBA for 2.5 h. Then the MBA-modified Au electrode was treated with 0.4 M EDC-0.1 M NHS for 0.5 h to convert the terminal carboxylic group to active NHS ester. After rinsing with water and PBS buffer and drying with nitrogen, the electrode surface was covered with 20.0 μL 550 μg mL^{-1} Sudan I Mabs PBS solution and incubated at 37 °C for 2 h. The unbound antibodies were removed from the electrode surface by slow rinsing with PBS buffer (pH 7.4) and water thoroughly. Then the antibody-modified electrode was treated with 1.0% BSA-PBS for 0.5 h to block the unreacted and nonspecific sites. Finally, the electrode was thoroughly rinsed with water and dried with nitrogen. The electrode was kept at 4 °C in a refrigerator for storage.

2.7. Preparation of samples

Three chili powder samples (sample A, B, C) were purchased from a local market. Spiked chili samples were prepared by spiking appropriate amounts (10 μg g^{-1} in sample A, 20 μg g^{-1} in sample B and 50 μg g^{-1} in sample C) of Sudan I into chili powder. All samples were dried and thoroughly homogenized before extraction. One gram of powder was exactly weighed and then ultrasonicated for 30 min with 10 mL of DMF, the mixture was centrifuged at 4500 rpm for 10 min.Then the supernatant was transferred for EIS detection.

2.8. Detection of Sudan I by EIS

10 μL sample extracts were added into 10.0 mL of 0.01 M PBS solution (pH 7.0) containing 2.5 mM $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ (1:1), then the immunosensor was dipped into the PBS solution and incubated at 37 °C for 1 h with constant temperature and humidity. Then EIS was used for the detection of Sudan I. The impedance measurements were performed at the frequency range from 10^{-1} to 10^5 Hz at the formal potential of 220.0 mV, using alternative voltage of 5.0 mV.

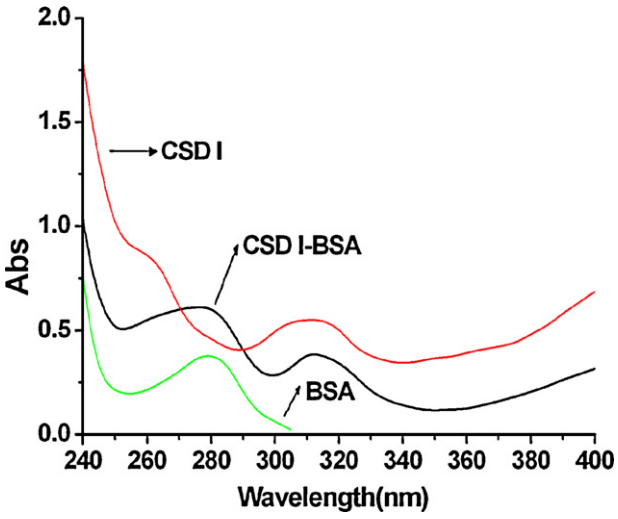

Fig. 2. UV–vis spectra of CSD I, BSA and CSD I-BSA.

2.9. HPLC-UV analysis

Sudan I standard solutions or sample extracts were passed through a 0.45 μm cellulose acetate membrane filter prior to HPLC detection. A HPLC system with a C18 column (150 mm × 4.6 mm, 5.0 μm particle size) was equilibrated with mobile phase consisting of methanol and 2% aqueous acetic acid (90:10, v/v) at a flow rate of 1 mL min^{-1}. The injection volume of standard or extract in each analysis was 20 μL. Sudan I was monitored at 478 nm by UV detector. The HPLC workstation software was used for the instrument control and data analysis. Peak areas were used for quantification.

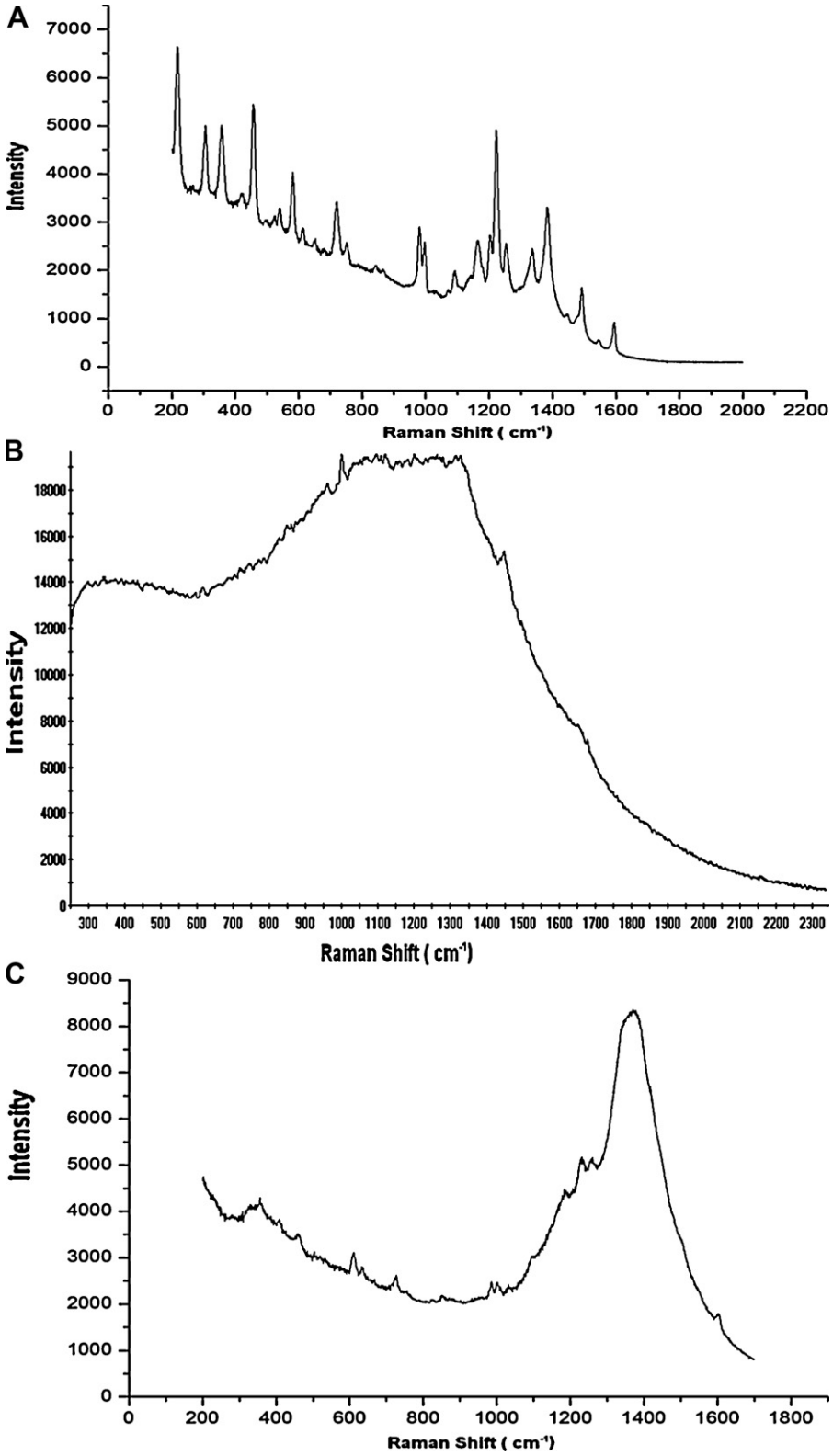

Fig. 3. Raman spectra of (A) CSD I, (B) BSA and (C) CSD I-BSA.

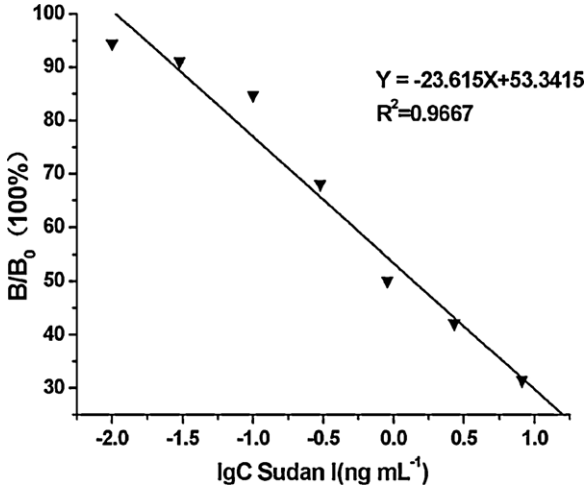

Fig. 4. Representative ELISA standard curve for Sudan I. Coating antigen concentration was 0.01 μg mL^{-1}, monoclonal antibody dilution = 1:1 × 10^5, goat anti-mouse IgG–HRP dilution = 1:5000.

3. Results and discussion

3.1. Characterization of immunogen by UV–vis and Raman spectra

By a peptide bond method, CSD I was conjugated to a carrier protein for immunizations. The individual UV–vis spectra for CSD I, BSA and CSD I-BSA are shown in Fig. 2, it can be seen that BSA and CSD I exhibited specific absorption peaks at 280 and 310 nm, while CSD I-BSA exhibited two specific absorption peaks at 280 and 310 nm, which indicated that the CSD I molecules were successfully coupled with BSA.

Fig. 3 shows the Raman spectra of CSD I (A), BSA (B), CSD I-BSA (C). As shown in curve A, the azo group of CSD I has a specific peak (intensity = 3500) at 1400 cm^{-1}. As compare to curve A, it can be seen that CSD I-BSA (curve C) also exhibited a strong peak at 1400 cm^{-1}, but the intensity increased to 8500 and the peak area increased distinctly, this suggests a large number of peptide bonds (1380–1550 cm^{-1}) was created by the coupling reaction between CSD I and BSA. In curve B, an intense broad peak was observed from 1000 to 1300 cm^{-1}, which was due to carboxyl groups and amino groups in the BSA molecules. While all peaks in the CSD I-BSA (curve C) were downshifted relative to BSA, and no predominant peaks can be observed from 1000 to 1300 cm^{-1}, this may be because the CSD I molecules were conjugated to the surface of the BSA, and covered the external groups of BSA. These results confirmed that the CSD I-BSA was produced in the conjugation reaction successfully.

3.2. Characterization of anti-Sudan I monoclonal antibody

The titer of anti-Sudan I monoclonal antibody was determined by checkerboard titration [30]. The optimal assay conditions of ELISA when the OD_{450} value was about 1.0 were chosen. The optimal concentration of the coating conjugate was found to be 20 μg mL^{-1}, the best dilution for antibody varied in the range of 1:60,000 to 1:100,000, suitable dilution of goat anti-mouse IgG-HRP was 1:5000. Under the optimal conditions, we found that the titer of the ascite antibody was 128,000.

Representative indirect competitive ELISA standard curve for Sudan I was presented in Fig. 4. The sensitivity was expressed by IC_{50} value (50% inhibition levels), the lower of the IC_{50} value, the higher sensitivity of the assay would be. As was shown in Fig. 4, the inhibition ratio showed a good linear relationship with logarithm of Sudan I concentration (R^2 = 0.9667) in the range of 0.01–8.1 ng mL^{-1}, with an IC_{50} value of 1.38 ng mL^{-1}. This result is comparable to the results in previous reports, with the IC_{50} value in the range of 0.3–2.0 ng mL^{-1} [28–32].

3.3. Preparation of the immunosensor

The schematic diagram of the immunosensor fabrication is presented in Fig. 5. First, a monolayer of MBA was formed on the electrode surface through the strong Au–thiolate bond. To provide high sensitivity and good repeatability in measurement, it is important to find suitable linker compounds to enable packing a high density of antibody on the electrode surface. Therefore, the next step is the activation of the SAM by the formation of NHS ester in the NHS-EDC solution [38,39]. After that, the active NHS esters were replaced by the primary amines of the Sudan I Mabs, and the Mabs was thus immobilized through the amide bond. Finally, a specific binding event occurred between the immobilized antibodies and Sudan I on the surface of the electrode.

3.4. Cyclic voltammetric characterization

CV can provide useful information on the changes of the electrode behavior after each assembly step. The CV measurement was taken from −0.2 V to 0.6 V with a scan rate of 100 mV s^{-1}

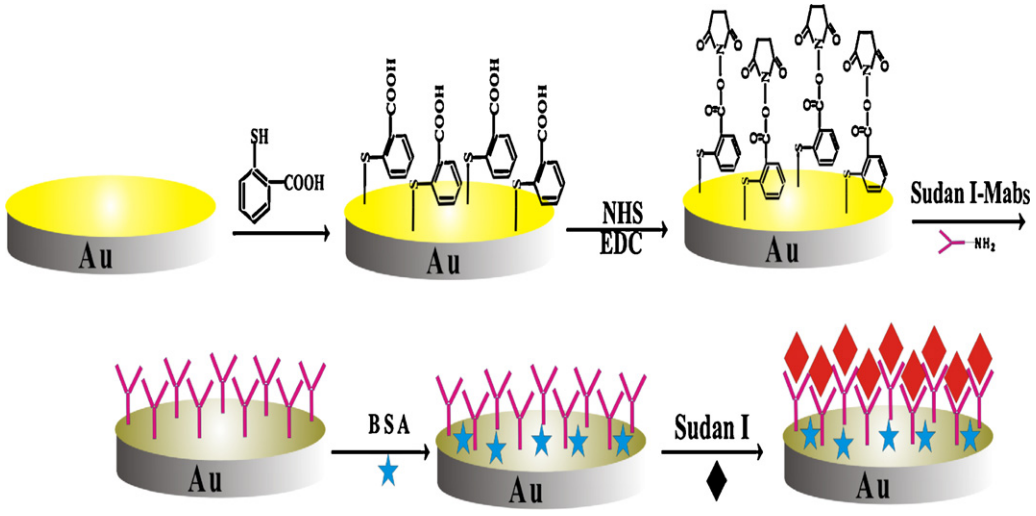

Fig. 5. Schematic illustration of the stepwise immunosensor fabrication process.

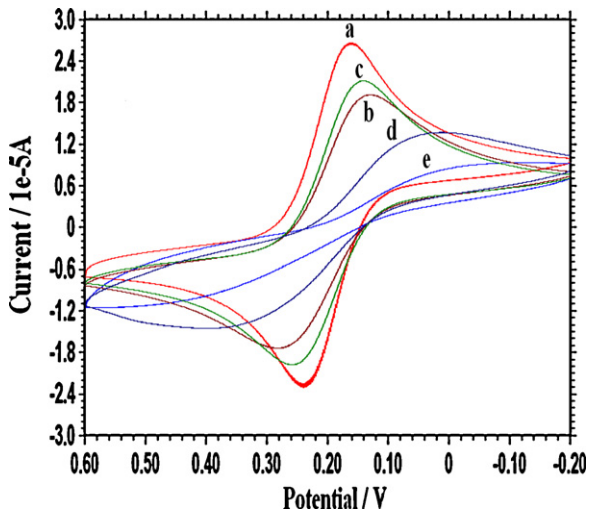

Fig. 6. Cyclic voltammetry of the Au electrode in the presence of 2.5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (scan rate 100.0 mV s^{-1}): (a) bare Au electrode; (b) after modified with MBA; (c) after activation with EDC/NHS; (d) after antibody immobilization; (e) after Sudan I (1 ng mL^{-1}) binding.

at room temperature. As shown in Fig. 6, the redox probe $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ reveals a reversible cyclic voltammogram at a bare Au electrode (curve a). After the pretreated Au electrode was covered with MBA, the formation of the MBA-monolayer on Au electrode resulted in a highly insulating surface and thus, blocked almost all the faradic currents (curve b) [40]. After activated by the co-addition of NHS and EDC, the negatively charged terminal carboxylic group of MBA was replaced by NHS ester. Due to electrostatic attraction, the positively/neutrally charged NHS ester promoted the transfer of the negative redox probe to the electrode surface. So an increased current response was observed (curve c). When antibody macromolecules were immobilized on the Au-SAM, the penetration of the redox probe was reduced. Therefore, the response current decreased (curve d). After the binding of Sudan I to the immobilized antibodies, the penetration of the redox probe was further reduced (curve e).

3.5. Optimal conditions for immunoreaction

The combination of Sudan I on the immunosensor could change the interface properties of electrodes, resulting in a change of Ret. Several factors, such as incubation temperature and incubation time, were investigated.

Table 1
EIS results from Fig. 7.

Concentration of Sudan I (ng mL^{-1})	Ret (Ω)	ΔRet (Ω)
0	1091	
0.05	1919	828
0.1	2758	1667
0.5	3969	2878
1	5228	4137
5	6595	5504
10	8132	7041
50	11,266	10,175

The effect of incubation temperature on the EIS value for the antibody–antigen reaction was studied in a temperature range of 20–60 °C. The modified gold electrode was immersed in 0.1 ng mL^{-1} Sudan I solution at different temperatures for 60 min. The electron-transfer resistance (Ret) was recorded by Faradic impedance measurement. The maximum response occurred at a reaction temperature of 37 °C, whereas temperatures over 37 °C resulted in a decrease of the Ret, as shown in Fig. 7A. The reason for this may be that the high temperature caused irreversible reactions such as dissociation and denaturation of antibody–antigen conjugate and thus affect the process. Additionally, temperatures lower than 37 °C could reduce the immunoreaction rate, thus lengthening the incubation time. Thus, 37 °C was selected in our work.

Incubation time also greatly affected the combination of Sudan I and Sudan I Mabs. The Sudan I Mabs and BSA modified gold electrode was immersed in 0.1 ng mL^{-1} Sudan I solution at 37 °C for different time periods. The effect of reaction time on the EIS response is shown in Fig. 7B. With the increase of reaction time, the electrochemical response of the immunological reaction increased and then reached a plateau when the reaction time was longer than 60 min. As a result, 60 min was chosen to be incubation time.

3.6. Calibration graph and limit of detection

Under optimized experimental parameters, the impendence spectra were obtained in PBS buffer by EIS. The corresponding Nyquist plots of impedance spectra are shown in Fig. 8, and the corresponding values of Ret are presented in Table 1. The ΔRet was calculated by following equation: $\Delta Ret = Ret_S - Ret_0$, where Ret_S and Ret_0 are signals in the presence and in the absence of Sudan I, respectively. Calibration curve for the immunosensor was shown in the inset of Fig. 8. The linear segment increases from 0.05 ng mL^{-1} to 50 ng mL^{-1} with a regression equation of $\Delta Ret = 2957.1 \lg c + 4268.1$ ($r^2 = 0.9677$, c in ng mL^{-1}, ΔRet in Ω), and the LOD is 0.03 ng mL^{-1} (3σ).

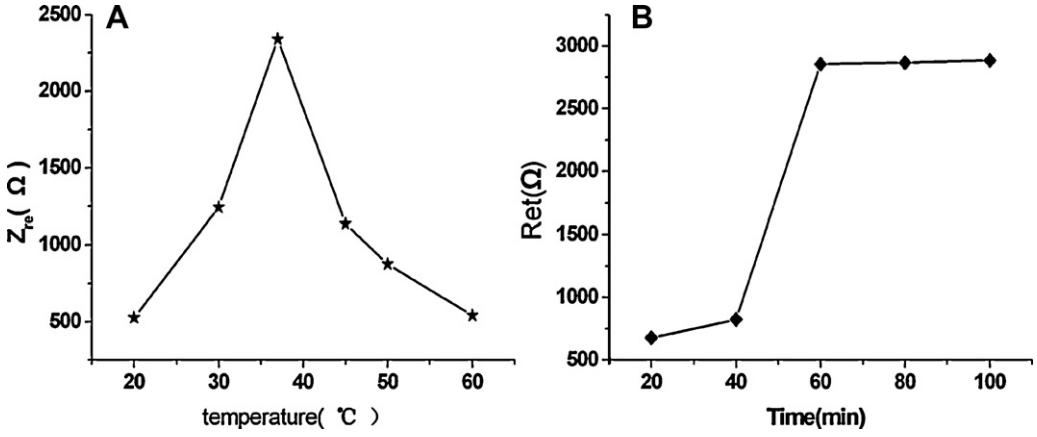

Fig. 7. Effect of incubation temperature (A) and immuno-reaction time (B).

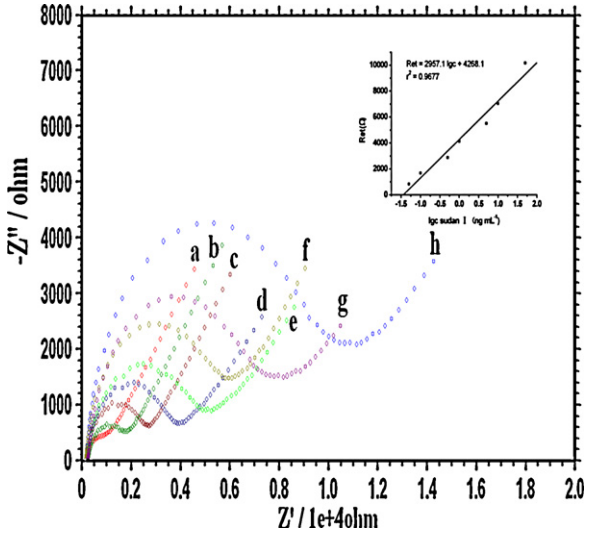

Fig. 8. Impedance spectra of the immunosensor with different concentrations of Sudan I on their surface in the presence of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ as a redox probe: (a) 0 ng mL^{-1}, (b) 0.05 ng mL^{-1}, (c) 0.1 ng mL^{-1}, (d) 0.5 ng mL^{-1}, (e) 1 ng mL^{-1}, (f) 5 ng mL^{-1}, (g) 10 ng mL^{-1}, (h) 50 ng mL^{-1}.

3.7. Sudan I assay in real samples

After 10 μL of the extract solution from the hot chili powder samples was added into 10.0 mL of 0.01 M PBS solution (pH 7.0), the proposed immunosensor was employed to determine Sudan I in chili powder samples (sample A, B, C). Table 2 shows the content of Sudan I in different chili powder samples, which obtained by the standard addition method. Each sample undergoes three parallel measurements, and the RSD is 5.2%, 5.3% and 3.8%, respectively. No signal was observed for Sudan I in hot chili powder, therefore, the powder sample was spiked with Sudan I at a certain concentration, and then analyzed by the above-described procedure. The recovery of powder A, B and C is 107.3%, 101.7% and 96.5%, respectively. For comparison, the spiked samples were detected by the HPLC system. The recovery is 105.4%, 103.9% and 99.7%, respectively. Both methods were highly correlated (RE = 1.8%, 2.1% and 3.2%, $n = 3$), which indicated that this method has good accuracy and great potential in the practice sample analysis.

3.8. Specificity and stability of the immunosensor

The specificity of the immunosensor was investigated by measuring the cross-reactivity with the EIS using different azo dyes. The immunosensors were immersed in Sudan I, II, III, IV with same concentration of 1.0 ng mL^{-1}. After incubated for 1 h, impedance spectra were recorded. Fig. 9 indicates the electro-transfer resistance change after binding with different azo dyes. The ΔRet of immunosensor with Sudan I, II, III, IV was 4137 Ω, 145 Ω, 484 Ω, 95 Ω, and the cross-reactivity between the immmunsensor and Sudan II, III and IV is 3.5%, 11.7% and 2.3%, respectively, indicating that the possible presence of other related Sudan dyes did not provide significant interference.

Table 2
Determination of Sudan I in hot chili samples.

Sample	Spiked (μg g^{-1})	Found (μg g^{-1})	Recovery (%)	RSD (%) ($n = 3$)
Powder A	0.00	0.00	107.3	5.2
	10.00	10.73		
Powder B	0.00	0.00	101.7	5.3
	20.00	20.34		
Powder C	0.00	0.00	96.5	3.8
	50.00	48.26		

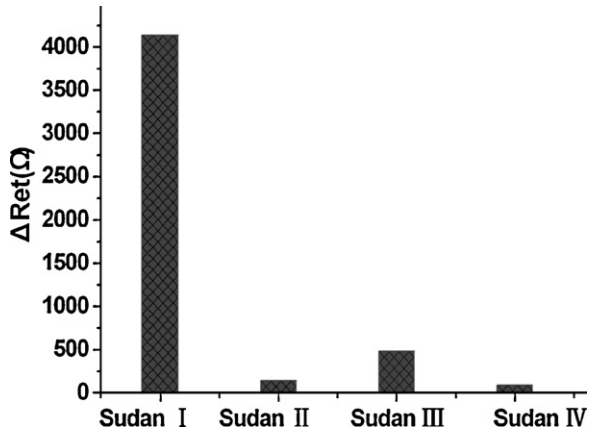

Fig. 9. Investigation of the immunosensor specificity. The concentration of the four different Sudan dyes is 1 ng mL^{-1}.

The stability of the immunosensor was evaluated by measuring the Ret responses at a fixed Sudan I concentration of 1 ng mL^{-1} over a period of 4 weeks. The immunosensor was investigated one assay a day and stored in refrigerator at 4 °C. The experimental results indicated that the Ret responses deviated only 10% ($n = 20$), revealing that the immunosensor fabricated by this method possesses long-term stability.

4. Conclusions

In this paper, an electrochemical impedance immunosensor for the detection of Sudan I was developed by covalently conjugating the Sudan I Mabs with MBA on the gold electrode. Co-addition of EDC and NHS could condense antibodies reproducibly and densely on the SAM. The Sudan I concentration was measured through the increase of impedance values in the corresponding specific binding of Sudan I and Sudan I antibody. The ΔRet values were proportional to the logarithmic value of Sudan I concentrations in range of 0.05–50 ng mL^{-1} with a detection limit of 0.03 ng mL^{-1}. The proposed method was proven to be a feasible quantitative method for Sudan I analysis with the properties of stability, highly sensitivity and selectivity.

Acknowledgements

We greatly appreciate the financial support of the National Nature Science Foundation of China (no. 20775026), Science and Technology Commission of Shanghai Municipality (no. 1052nm06500) and Shanghai Key Laboratory of Green Chemistry and Chemical Process.

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