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Development of A Multi-residue Immunosensor Based on an Anti-bovine Serum Albumin-clenbuterol-salbutamol Antibody for Detection of six *β*-agonists

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The multi-determinant antigen bovine serum albumin (BSA)-clenbuterol (CL)-salbutamol (SAL) was prepared and the anti-BSA-CL-SAL antibody was obtained by animal immunization. Furthermore, the multi-residue immunosensor modified by graphene (PG)/chitosan (CTS)/CL was developed. The PG/CTS/CL complex was characterized by UV-Vis. The electrochemical properties of the modified electrode were studied by cyclic voltammetry and electrochemical impedance spectroscopy. Six *β*-agonists (CL, SAL, ractopamine (RAC), terbutaline (TER), mabuterol (MAB) and tulobuterol (TUL)), were detected by indirect competitive method. The lowest detection limits were 0.2, 0.1, 0.1, 0.4, 0.1, 0.1 ng·mL^{-1}, respectively, and the linear ranges were 100-5000, 50-7000, 500-7000, 10-6000, 100-6000, 100-6000 ng·mL^{-1}, respectively. PG/CTS/CL multi-residue immunosensor has good repeatability and stability.

Keywords: anti-BSA-CL-SAL antibody; *β*-agonists; detection; multi-residue immunosensor

1. INTRODUCTION

β-agonists, commonly known as "Clenbuterol", are the general name of a class of *β*-receptor stimulants [1]. They are phenylethanolamine derivatives, which are linked with alkaline secondary amine side chains on the benzene ring. According to different substituents, they can be divided into aniline and phenol [2]. Aniline mainly includes Cl, SAL, etc. [3], and phenol mainly includes RAC, TER, etc. [4]. After being eaten by animals as feed, it can promote protein synthesis in the metabolic process and inhibit fat production in animals. The feed conversion rate, growth rate and carcass lean meat rate can be increased by more than 10% [5,6]. After eating meat containing *β*-agonists, people will have heart

palpitations, limb muscle fibrillation [7], hand shaking or even unable to stand, dizziness, fatigue and other abnormalities [8,9]. It is more dangerous for patients with sympathetic hyperfunction, especially for patients with diseases such as hypertension, heart disease, hyperthyroidism and prostate hypertrophy [10,11]. Use *β*-agonists in China and most countries in the world are forbidden, which are the residues that must be detected in animal derived foods. This puts forward high requirements for the sensitivity of residue detection. Therefore, highly sensitive multi-residue immunoassay for the detection of *β*-agonists is an urgent technology in this field.

Gas chromatography and high-performance liquid chromatography have been applied to the fields of food safety and environmental monitor, which can realize the qualitative and quantitative analysis of *β*-agonists [12-14]. However, the disadvantages of cumbersome operation, high detection cost, complex sample pretreatment and long detection cycle make it difficult to be popularized in a wide range, especially not suitable for on-site rapid detection. Traditional immunoassay methods can only detect one analyte, and there are many kinds of *β*-agonists and often exist at the same time [15]. The contradiction between the two is the main bottleneck in the application of immunoassay in food rapid detection technology. There is an urgent need for an immunoassay method that can analyze multiple or one class of hazards at one time. Multi-residue immunoassay, i.e. multi-component analyte immunoassay, refers to the method of simultaneous determination of two or more related analytes in the same sample [16]. The establishment of multi-residue immunoassay technology is mainly to prepare an antibody that can recognize a variety of analytes: wide spectrum specific antibody [17]. Multiple haptens are coupled to the same carrier protein to prepare antigen (multi-determinant antigen) for antibody preparation, which becomes a suitable choice for preparing wide spectrum specific antibody.

Electrochemical immunosensor is a new type of sensor combining highly sensitive electrochemical technology and highly specific immune technology. It is a detection technology with high speed, high sensitivity, simple operation and low cost [18.19], and has a wide application prospect in food, environment and other domains [20]. In this paper, multi-determinant antigen BSA-CL-SAL and broad-spectrum specific anti-BSA-CL-SAL antibody were prepared and combined with electrochemical technology to form a multi-residue electrochemical immunoassay method, which has more simple operation, higher speed and higher sensitivity, and can realize the rapid multi-residue detection of *β*-agonists with important practical significance.

2. EXPERIMENTAL SECTION

2.1. Reagents

Ractopamine (RAC), clenbuterol (CL), salbutamol (SAL), terbutaline (TER), mabuterol (MAB) tulobuterol (TUL) and bovine serum albumin (BSA) were purchased from National Institutes for Food and Drug Control. Ovalbumin (OVA) was obtained from Sinopharm Chemical Reagent Co., Ltd. Phosphate buffer solution (0.1 M, pH=7.4) was prepared from KH_2PO_4 and NaOH. All other chemicals and reagents were of analytical grade.

2.2. Instruments

Electrochemical propety of PG/CTS/CL immunosensor was measured by a CHI600E electrochemical workstation (Shanghai Chenhua Apparatus Co., Ltd, China). The experiments were performed by a three-electrode system with platinum (Pt) wire, Ag/AgCl, and glassy carbon as counter electrode, reference electrode, and working electrode, respectively. UV-Vis absorption spectra were monitored by UV-1700PC spectrophotometer (Shanghai Fenghuang Optical Instrument Co., Ltd, China).

2.3. Preparation of multi-determinant antigen BSA-CL-SAL and anti-BSA-CL-SAL antibody

143.6 mg SAL was weighed and reacted with 68 mg glutaric anhydride at 25℃ for 6 hours to get salbutamol hemisuccinate monoester solid. After dissolving in the mixed solvent of dimethylformamide (DMF) and 1,4-dioxane, tri-*n*-butylamine(TBA) and isobutyl chloroformate(IBCF) were added and reacted for an hour. The solution was added into the PBS solution of cold BSA-CL conjugate [21], and the BSA-CL-SAL antigen was prepared.

BSA-CL-SAL antigen was prepared into standard solution and injected into healthy rabbits, which was injected again to strengthen the immunity after a period of time. After three months of immune reaction, rabbit serum was isolated to obtain anti-BSA-CL-SAL antibody.

2.4. Preparation of PG/CTS/CL multi-residue immunosensor

4 μL PG/CTS suspension [22] and 2 μL 0.2 $g{\cdot}L^{-1}$ clenbuterol standard solution were applied dropwise on the surface of the polished electrode, then dried at 37℃. The electrode was incubated in 0.05% OVA solution for reducing nonspecific adsorption. The electrode was washed with phosphate buffer solution (pH=7.4) to prepare PG/CTS/CL multi-residue immunosensor.

2.5. Characterization

UV-Vis: The modification process of electrode surface was simulated by taking clean quartz glass slides. 600 μL PG/CTS suspension and 300 μL 0.2 $g{\cdot}L^{-1}$ clenbuterol standard solution were applied dropwise on the quartz glass slides, then dried at 37℃. Scrape it off with a clean blade and disperse it in distilled water. Wavelength scanning range: 190–800 nm, scanning interval: 0.1 nm.

All electrochemical experiments used three electrode system. The cyclic voltammetry (CV) was measured with scanning range of –0.2~0.8 V, scanning rate of 100 $mv{\cdot}s^{-1}$ in PBS solution of 2 mM potassium ferricyanide / potassium hexacyanoferrate (II).

The EIS was determined with scanning frequency of 0.01~10^6 Hz, pulse amplitude of 5 $mV{\cdot}s^{-1}$ in 0.1 M potassium chloride, 5 mM potassium ferricyanide, and 5 mM potassium hexacyanoferrate (II) solution.

2.6. Optimization of condition

The amount of antibody was optimized: In 50 μL incubation solution (anti-BSA-CL-SAL antibody and phosphate buffer solution), and added different volume of antibodies. The immunosensor was immersed in incubation solution for 30 min. After incubation, the electrode surface was washed with phosphate buffer solution, and analyzed by DPV.

Optimizing the incubation time: The optimum volume of anti-BSA-CL-SAL antibody was added to the incubation solution (anti-BSA-CL-SAL antibody and phosphate buffer solution) with a total volume of 50 μL. The electrode was incubated, and measured the peak current of DPV every 5 min.

2.7. Detection of six β-agonists

Placing the immunosensor in 50 μL series of *β*-agonist incubation solution (*β*-agonist, quantitative antibody and phosphate buffer solution), and incubated at 37℃ for the optimum time. The electrode surface was washed with phosphate buffer solution, and determined by DPV in phosphate buffer solution of 2 mM potassium ferricyanide/ potassium hexacyanoferrate (II).

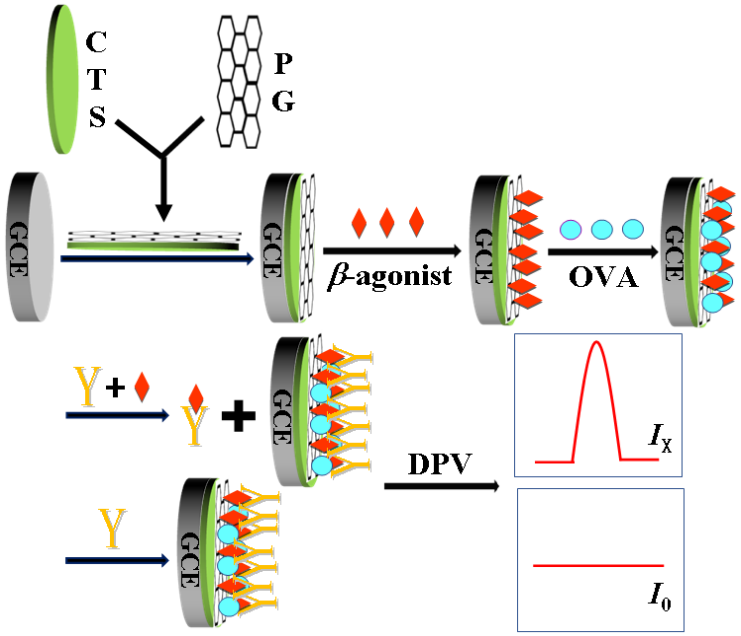

Figure 1. Schematic illustration for preparation and application of the immunosensor

3. RESULTS AND DISCUSSION

3.1. Detection principle of PG/CTS/CL multi-residue immunosensor

The process of the prepared immunosensor with PG/CTS complex and the detection principle of the sensor are shown in Fig.1. The prepared immunosensor was immersed in the solution of *β*-agonist

and anti-BSA-CL-SAL antibody. The *β*-agonist in solution competes with the *β*-agonist fixed on the surface of the immunosensor to bind the quantitative antibody in solution. The antibody content bound to the appearance of the immunosensor is not proportional to the *β*-agonist content in solution. The antibody bound to the electrode impedes the transmission of electrons on the electrode surface, resulting in the decrease of DPV response current. The decrease degree of response current is proportional to the antibody content on the electrode, that is, the response current value is directly proportional to the *β*-agonist content in solution. The incubation solution containing only antibodies was used as the blank sample. The quantitative relationship between DPV peak current difference ($\Delta I=I_x-I_0$, I_x as the sample detection corresponding peak current, I_0 as the blank sample detection corresponding peak current) and *β*-agonist concentration was established.

3.2. UV-Vis of PG/CTS/CL complex

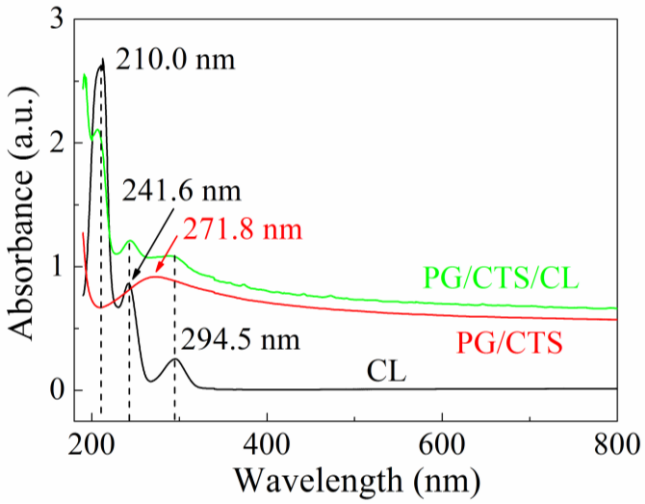

Figure 2. UV-Vis absorption spectra of CL, PG/CTS and PG/CTS/CL complex (600 μL PG/CTS suspension and 300 μL 0.2 g·L^{-1} clenbuterol standard solution, wavelength scanning range: 190–800 nm, scanning interval: 0.1 nm, absorbance range: -3.0-3.0)

For further studying the interaction between PG/CTS/CL complexes on the electrode surface, the UV-Vis spectra of clenbuterol (CL)、PG/CTS and PG/CTS/CL complexes were studied, as shown in Fig. 2. It can be seen from Fig. 2 that clenbuterol (CL) standard solution has three characteristic absorption peaks at 210.0 nm, 241.6 nm and 294.5 nm, and PG/CTS complex has a wide range of characteristic absorption peak at 271.8 nm. The PG/CTS/CL complex shows clenbuterol (CL) absorption peaks at 210 nm and 241.6 nm. The absorption peak of clenbuterol (CL) at 294.5 nm interferes with the wide absorption peak of PG/CTS at 271.8 nm, resulting in a "platform" absorption peak in the range of 271.8~294.5 nm, which is the composite peak of clenbuterol (CL) and PG/CTS, which proves that clenbuterol (CL) has been modified on the surface of PG/CTS [23].

3.3. Electrochemical characterization of PG/CTS/CL immunosensor

In the process of electrode modification, CV was used to characterize the changes of electrochemical properties on the electrode surface, as shown in Fig. 3A. It can be seen from Fig. 3A that the bare electrode shows a pair of reversible and obvious ferricyanide ion/ferrocyanide ion redox peaks (curve a), indicating that the electrode surface was cleaned. After modifying PG/CTS and CL complexes, the redox current response of the electrode is significantly enhanced than that of the bare electrode (curve b), which is due to the excellent conductivity of PG increasing the electron transfer rate on the electrode surface. After antibody immunization, the peak current decreased significantly compared with the modified electrode (curve c), which was caused by the antibody blocking the transfer of the electron on the surface of the electrode. CV curves show that CL was successfully modified on the electrode [23].

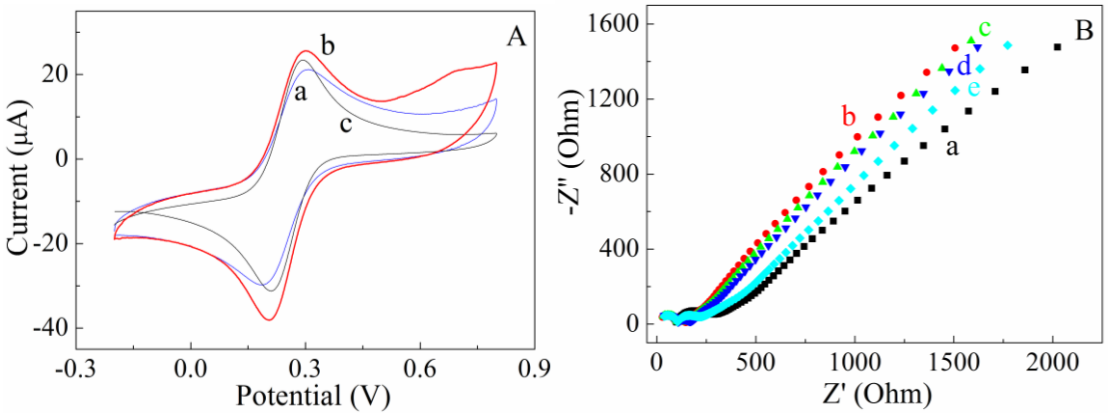

Figure 3. (A) CV curves for (a) glassy carbon electrode, (b) PG/CTS/CL, and (c) anti-BSA-CL-SAL antibody immunization. (In 2 mM $K_3[Fe(CN)_6]/K_2[Fe(CN)_6]$ buffer solution of PBS (c=0.1 mol/L, PH=7.4), the scanning range was -0.2-0.8V, the scanning rate was 100 mv/s); (B) EIS curves for (a) glassy carbon electrode, (b) PG/CTS, (c) PG/CTS/CL, (d) PG/CTS/CL/OVA, and (e) anti-BSA-CL-SAL antibody immunization. (Electrolyte solution: KCl (0.1 mol/L), $K_3[Fe(CN)_6]$ (0.005 mol/L) and $K_4[Fe(CN)_6]$ (0.005 mol/L). Glassy carbon electrode was used as working electrode, platinum wire as counter electrode, and Ag/AgCl electrode as reference electrode. Scanning Frequency: Low Frequency: 0.01 Hz, High Frequency: 10^6 Hz; Amplitude: 0.005V; Waiting time: 2 s)

The EIS of different modified electrodes is shown in Fig. 3B. The impedance value of bare glassy carbon electrode is about 200 Ω (curve a). After modifying PG/CTS, due to the excellent conductivity of PG/CTS complex, the electron transfer rate on the electrode surface is accelerated and the impedance value is significantly reduced, about 50 Ω (curve b). The impedance value hardly changed after modifying CL, indicating that CL has no obvious effect on the electron conduction rate on electrode surface. After OVA block the site, OVA have a great impact on the electron conduction rate. However, since most of active sites of PG/CTS complex on electrode surface have been bound by CL, OVA are less bound to the electrode surface and have little effect on the electron transfer rate. The impedance

value is about 65 Ω (curve d). After antibody immunization, the antibody with poor conductivity binds to the CL modified on electrode surface, and the impedance value increases significantly, about 130 Ω (curve e), hindering the electron transfer on electrode surface.

3.4. Optimization of condition

In order to eliminate the influence of antibody volume and incubation time on the performance of PG/CTS/CL immunosensor in immune reaction, the antibody volume and incubation time were optimized at 37℃, as shown in Fig. 4. Fig. 4A shows that when the antibody amount is in the range of 2~11 μL, the DPV peak current decreases with the increase of antibody amount, and then increases slightly, indicating that when the antibody amount is 11 μL, the antibody binding amount basically reaches saturation. According to the principle of meeting the best detection requirements of the sensor and using the minimum antibody amount, the antibody volume is selected as 11 μL in the later detection. Fig. 4B shows that in the competitive reaction, the binding amount of antigen and antibody increases with the increase of incubation time. The incubation time is in the range of 5~15 min, and the DPV peak current decreases with the increase of incubation time, and then increases slightly, indicating that the binding amount of antibody basically reaches saturation when the incubation time is 15 min, so the incubation time is selected as 15 min. (All data in Fig.4 was from our research, and belong to us)

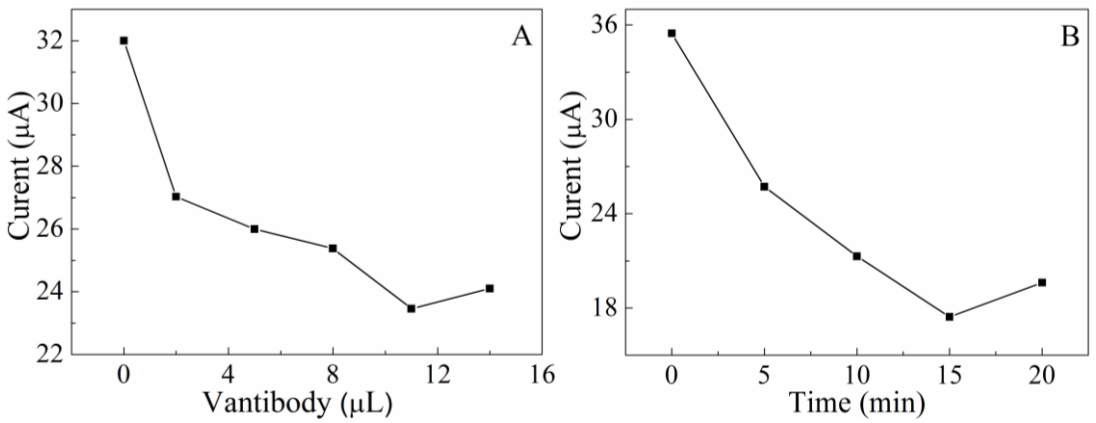

Figure 4. Effect of (A) anti-BSA-CL-SAL antibody volume and (B) incubation time on the DPV peak current. (All DPV data was measured in a 2 mM $K_3[Fe(CN)_6]/K_2[Fe(CN)_6]$ buffer solution of PBS (c=0.1 mol/L, PH=7.4) with a voltage range of −0.2-0.5 V and a pulse amplitude of 50 mV)

3.5. DPV detection of six β-agonists

In order to realize the quantitative detection of immunosensor, PG/CTS/CL immunosensor was used for the quantitative DPV detection of six *β*-agonists (ractopamine (RAC), clenbuterol (CL), salbutamol (SAL), terbutaline (TER), mabuterol (MAB), tulobuterol (TUL)) standard solutions, as shown in Fig. 5.

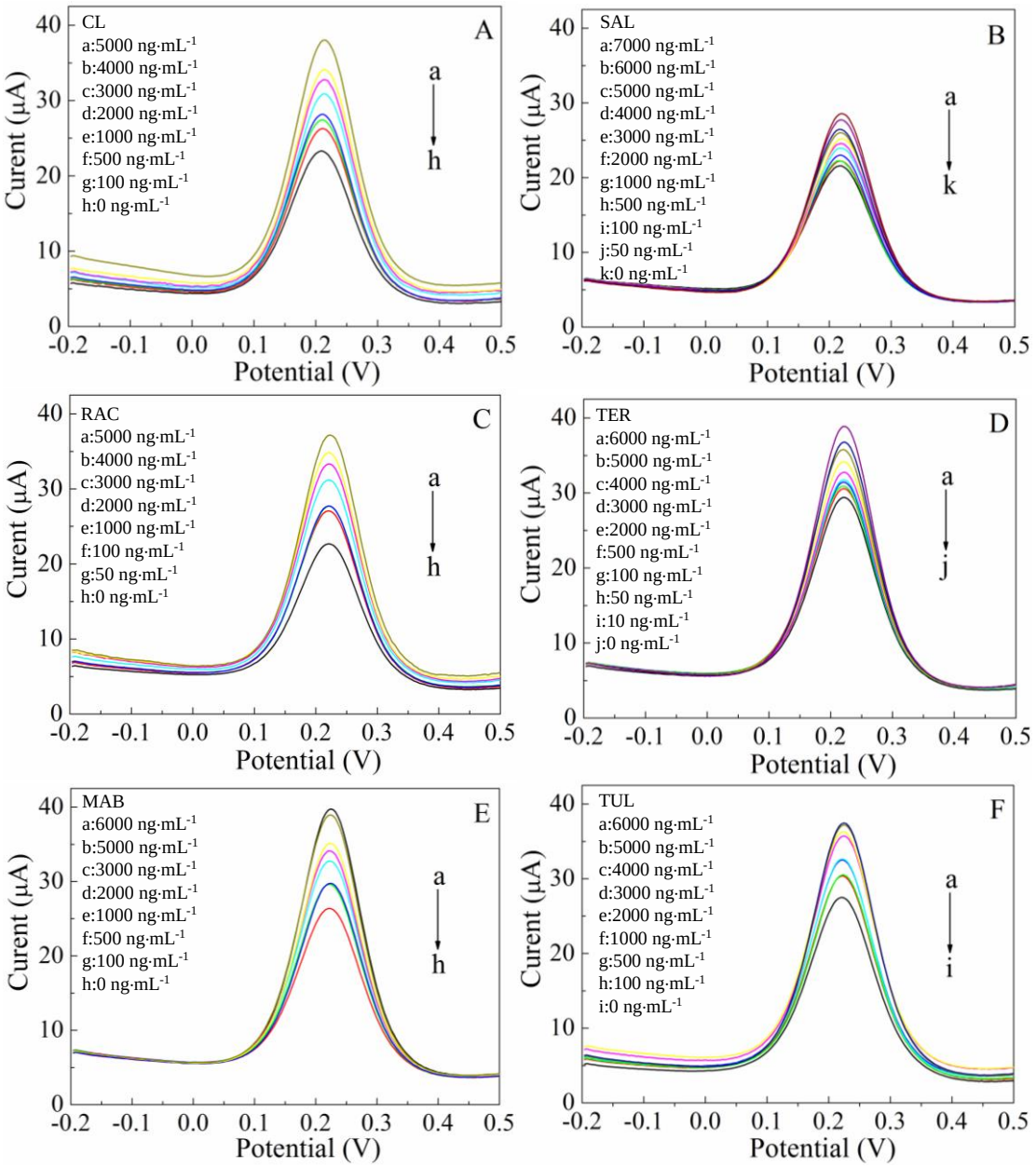

Figure 5. (A) CL detected by the PG/CTS/CL immunosensor (a, b, c, d, e, f, g and h corresponding to PG/CTS/CL, concentrations from 5000, 4000, 3000, 2000, 1000, 500, 100 and 0 ng·mL^{-1}, respectively). (B) SAL detected by the PG/CTS/CL immunosensor (a, b, c, d, e, f, g, h, i, j and k corresponding to concentrations from 7000, 6000, 5000, 4000, 3000, 2000, 1000, 500, 100, 50 and 0 ng·mL^{-1}, respectively). (C) RAC detected by the PG/CTS/CL immunosensor (a, b, c, d, e, f, g and h corresponding to concentrations from 5000, 4000, 3000, 2000, 1000, 100, 50 and 0 ng·mL^{-1}, respectively). (D) TER detected by the PG/CTS/CL immunosensor (a, b, c, d, e, f, g, h, i and j corresponding to concentrations from 6000, 5000, 4000, 3000, 2000, 500, 100, 50, 10 and 0 ng·mL^{-1}, respectively). (E) MAB detected by the PG/CTS/CL immunosensor (a, b, c, d, e, f, g and h corresponding to concentrations from 6000, 5000, 3000, 2000, 1000, 500, 100 and 0 ng·mL^{-1}, respectively). (F) TUL detected by the PG/CTS/CL immunosensor (a, b, c, d, e, f, g, h and i corresponding to concentrations from 6000, 5000, 4000, 3000, 2000, 1000, 500, 100 and 0 ng·mL^{-1}, respectively). All DPV data was measured in a 2 mM $K_3[Fe(CN)_6]/K_2[Fe(CN)_6]$ buffer solution of PBS (c=0.1 mol/L, PH=7.4) with a voltage range of −0.2-0.5 V and a pulse amplitude of 50 mV

From the DPV diagram: (1) after incubation, the DPV peak current of PG/CTS/CL immunosensor decreased significantly compared with the modified electrode, because the amount of anti-BSA-CL-SAL antibody specifically bound to the electrode surface gradually increased, which hindered the transmission of electrons. (2) With the decrease of *β*-agonist concentration, the DPV peak current decreases gradually.

This is because the sensor adopts the competition principle. The decrease degree of DPV peak current is inversely proportional to the concentration of *β*-agonist in solution. The detection results of PG/CTS/CL multi-residue immunosensor for six *β*-agonists standard solutions are shown in Table 1. SAL is the widest linear range and CL is the narrowest. Compare with other research which was already published, our new immunosensor shows a wider linear range and lower detection limit [23].

Table 1. Detection results of six *β*-agonists by PG/CTS/CL immunosensor

β-agonist	Linear range ($ng \cdot mL^{-1}$)	R^2	LOD ($ng \cdot mL^{-1}$)
CL	100~5000	0.995	0.2
SAL	50~7000	0.990	0.1
RAC	500~7000	0.985	0.1
TER	10~6000	0.990	0.4
MAB	100~6000	0.985	0.1
TUL	100~6000	0.981	0.1

Anti-BSA-CL-SAL antibody is a polyclonal antibody with wide spectrum specificity, and CL or SAL as antigenic determinant is similar to RAC, TER, MAB and TUL. Therefore, anti-BSA-CL-SAL antibody can recognize a variety of *β*-agonists and is the basis for multi-residue detection of the immunosensor.

PG/CTS/CL multi-residue immunosensor has better detection effect on SAL, wider linear range and lower detection limit than other *β*-agonists. PG/CTS complex contains a large number of carboxyl groups, hydroxyl groups and amino groups, as well as the π-π conjugate structure of PG, so that CL can stably bind to the surface of PG/CTS complex. CL is similar to SAL in structure. Therefore, CL has a good competitive relationship with SAL, which shows a good detection performance. The prepared PG/CTS/CL multi-residue immunosensor can detect six *β*-agonists, and displays a wider linear range and lower detection limit, which achieves the expected goal of multi-residue detection. (This paragraph explained the advantages compared with our research which published in ACS Omega 2020, 5, 10, 5548–5555. In that paper, each one immunosensor only can detect three different β-agonists, in this paper our further research prepared a new immunosensor which can detect six β-agonists at the same time, also has wider linear range and lower detection limit.)

3.6. Repeatability and stability

After the preparation of PG/CTS/CL immunosensor, the current response was measured continuously for 20 times under the same conditions. The standard deviation was 7.9%, indicating that the prepared PG/CTS/CL immunosensor has good repeatability. After the preparation of PG/CTS/CL immunosensor, it was stored at 4℃, and measured the current response three times a day for 7

consecutive days. The relative standard deviation was 8.5%, indicating that the prepared PG/CTS/CL immunosensor has good stability for a relatively long time.

3.7. Analysis of real samples

In order to evaluate the detection effect of the constructed immunosensor on the real sample, the prepared immunosensor was applied to the real sample detection. The PG/CTS/CL immunosensor was used as the investigation object, and clenbuterol was selected as the β -agonist to be detected.

Three samples of pig lean meat, pig fat and pig liver were selected, each with 1±0.005 g, and clenbuterol standard solution was quantitatively added. After labeling, 3mL extract (acetonitrile: acetone =1:1) was added to each extract, ultrasonic extraction for 20 min, centrifugation for 5 min, supernatant was taken and blow-dried with nitrogen, then fixed volume with 1mL PBS, and stored in a refrigerator at 4℃ for later use. Table 2 shows the PG/CTS/CL immunosensor detection results.

According to the detection results of real samples by PG/CTS/CL immunosensor, the recoveries of three different concentrations of three actual samples detected by the immunosensor were in the range of 82.6%-109.9%, and the relative standard deviations were in the range of 2.2%-11.6%. The detection results of clenbuterol in pig lean meat, pig fat and pig liver were satisfactory.

Table 2. detection results of CL in real sample by PG/CTS/CL immunosensor

Real sample	Amount added ($ng \cdot mL^{-1}$)	Amount measured ($ng \cdot mL^{-1}$)			RSD (%)	Recovery (%)
Pig lean	500	531.2	567.9	457.8	10.8	103.8
	2000	1898.2	2026.6	2017.5	3.6	99.0
	6000	5090.9	5054.2	4815.7	2.9	83.1
Pig fat	500	522	448.7	430.3	10.3	93.4
	2000	1834	1769.8	1733.1	2.8	88.9
	6000	5008.3	5026.6	4824.7	2.2	82.6
Pig liver	500	485.4	512.9	448.7	6.6	96.5
	2000	2035.9	2393.7	2164.3	8.2	109.9
	6000	6755.1	6553.3	5402.8	11.6	104.0

3.8. Advantages of PG/CTS/CL immunosensor compared with other published researches

There are many researchers and teams have published papers about immunosensors for detecting β-agonists, such as Qi based on graphene has developed a new type of rapid determination of ractopamine in the electrochemical immunosensor lake, the sensor has good stability, selectivity and repeatability, this research use ractopamine antibody competitive immune response, and ractopamine can effectively determine the free ractopamine concentration in the solution, at 0.1-10 ng mL^{-1} and 10 - 4000 $ng \cdot mL^{-1}$, respectively [24], and He used chitosan stabilized gold nanoparticles modified electrode to conduct electrochemical immune analysis of ractopamine. Ractopamine antibody was easy to adsorb on the surface of chitosan due to its high bio affinity. Chitosan could amplify the electrochemical signal of the electrode, and the detection limit was as low as 2.3 $pg \cdot mL^{-1}$ and detection range: 0.01-5 $ng \cdot mL^{-1}$ [25], another example is Ji prepared clenbuterol ultra-sensitive immunosensor by introducing the interaction between MoS_2-AuPt nanocomplex and biotin-streptavidin. The use of MoS_2-AuPt

accelerated electron transfer, which resulted in a sharp increase in the electrochemical signal of the electrochemical probe hydrogen peroxide. Under the optimal conditions, the response signal of the electrochemical immunosensor electrode showed a linear relationship with the concentration range of 10 pg·mL^{-1} to 100 ng·mL^{-1} clenbuterol, and the detection limit was as low as 6.9 pg·mL^{-1} [26]. Without exception, these immunosensor can only specifically detect a single β-agonist, comparing with those so many researches, our immunosensor's advantages is researchers can use one immunosensor to detect six different β-agonists at the same time, the workload of the researchers was greatly reduced. At the same time, our immunosensor's linear range and detection limit is very wide and low, and enough to meet most detecting requirements in real life.

4. CONCLUSIONS

Based on multi-determinant antigen BSA-CL-SAL and anti-BSA-CL-SAL antibody, PG/CTS/CL multi-residue immunosensor was developed. Under the optimal conditions, the detection of six *β*-agonists (ractopamine (RAC), clenbuterol (CL), salbutamol (SAL), terbutaline (TER), mabuterol (MAB) tulobuterol (TUL)) was realized, which display a low detection limit (0.1~0.4 ng·mL^{-1}) and a wide linear range (100~5000, 50~7000, 500~7000, 10~6000, 100~6000, 100~6000 ng·mL^{-1}, respectively). It has good repeatability (standard deviation 7.9%) and stability (standard deviation 8.5%), which achieve the expected goal of multi residue detection.

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