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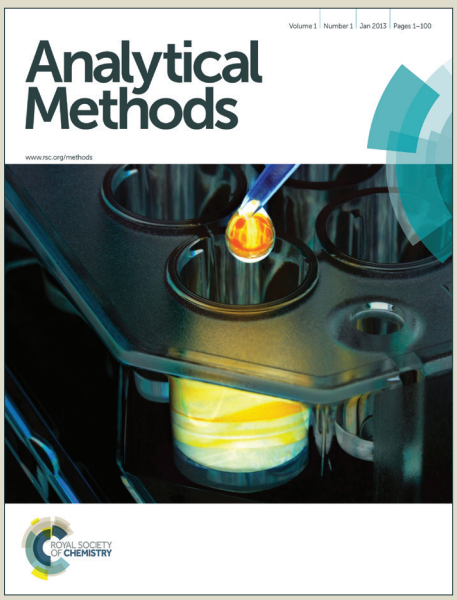

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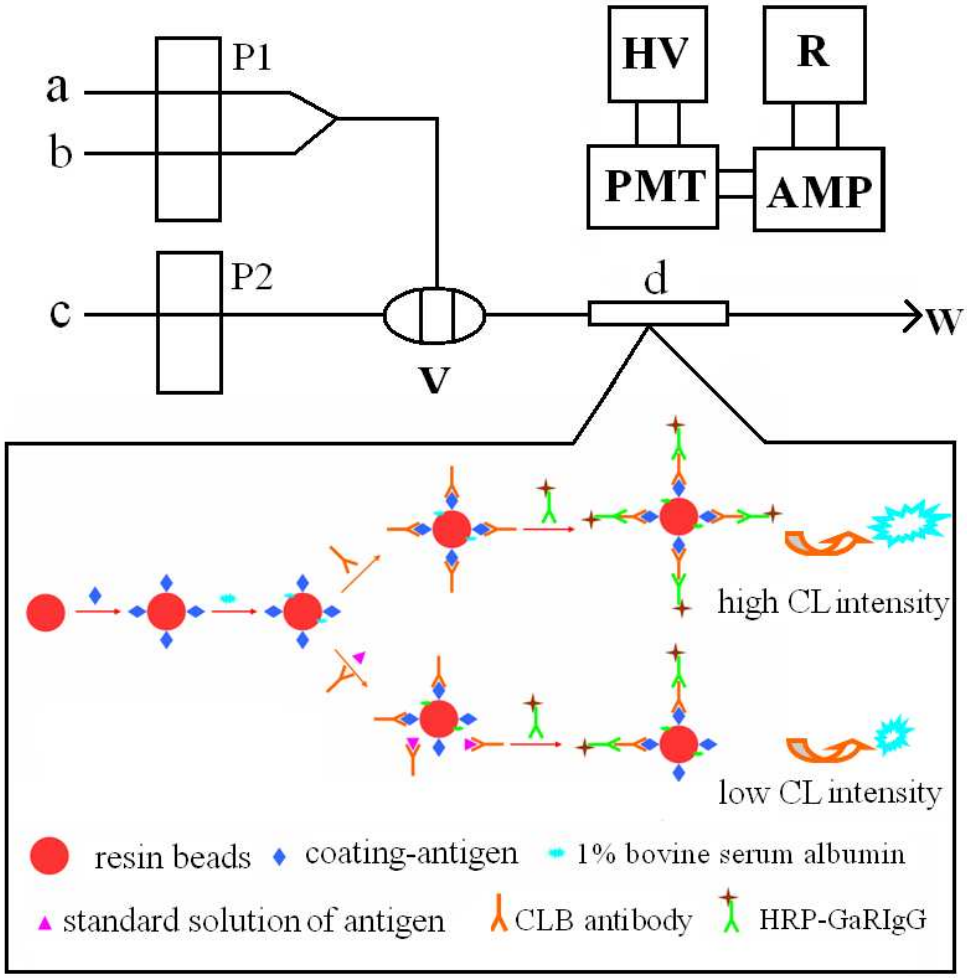
Graphical Abstract



Flow injection chemiluminescence immunosensor for the determination of clenbuterol by immobilizing coating-antigen on carboxylic resin beads

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Abstract:

In this paper, a flow-injection chemiluminescence (CL) immunoassay based on transparent immunoaffinity reactor has been developed for the detection of clenbuterol (CLB). The reactor prepared with coating-antigen immobilized carboxylic resin beads was used as an immunosensor which is grounded on the CL reaction system of the *p*-iodophenol-luminol-H_2O_2 in the presence of horse radish peroxidase (HRP) as an enzyme tracer. With a competitive immunoassay format, the decrease in CL intensity is proportional to the increase of clenbuterol concentration in the range of 0.40 – 120 ng mL^{-1} with a correlation coefficient of 0.999 (n=9) and a limit of detection (LOD) of 0.20 ng mL^{-1} at S/N of 3. The immunosensor system shows good stability, specificity and fabrication reproducibility. It has been applied to detecting practical samples with satisfactory results, which will exhibit a great prospect for screening of trace amount of CLB residue.

Keywords: Flow-injection analysis; Chemiluminescence; Immunosensor; Clenbuterol; Carboxylic resin beads

Introduction

Clenbuterol (4-amino-3, 5-dichloro-α-tert-butylaminomethylbenzyl alcohol hydrochloride, CLB), a well known β-adrenergic agonists, has been used as bronchodilators to treat respiratory diseases [1, 2]. CLB enhances the lean meat/fat ratio and increases the efficiency of feed conversion by inhibiting fat synthesis, improving muscular mass, and decreasing adipose tissue deposition in livestock production at a dose ten to hundred times higher than clinical dosage [3]. The produced meat may contain residues of agonists, which cause great harm to the human body. Various intoxications have been described as being due to the consumption of meat products containing clenbuterol residue [4, 5]. A large number of poisoning incidents caused by abusing clenbuterol additives have aroused people's attention. As a result, it has been prohibited as a therapeutic medicine in food-producing animals in many countries. Consequently, many countries in the world forbid it to be used in food-producing animals, and stipulate that the residues of clenbuterol can't be detected in the food products of animal origin. It is very important to develop a rapid, simple, accurate and sensitive method for the detection of CLB in order to ensure food safety and public health.

There are sensitive analytical methods for the detection of CLB, such as capillary electrophoresis (CE) [6], gas chromatography (GC) [7], liquid chromatography (LC) [8], surface enhanced Raman scattering (SERS) [9], electrochemiluminescent [10, 11] and highly sensitive immunoassays enzyme-linked immunosorbent assay (ELISA) [12, 13]. These methods have been developed to control their illegal use in meat products. However, the complicated and expensive instrument, such as LC, is not appropriate for the manipulation of average investigators. SERS is time-consuming and laborious, which is not suitable for screening large quantities of real samples.

Flow injection analysis (FIA) has been proven to be one of the most powerful techniques

for the determination of biological matrices. It possesses many advantages, such as being simple, less time consuming and easy to automate for high sample throughput. Therefore, it has been widely applied in various fields, including environmental monitoring [14], food safety [15], pharmaceutical analysis [16, 17], identification of bacteria [18] and clinical diagnosis [19, 20]. The combination of FIA detection with chemiluminescence [21-23] will be one of the most useful detection techniques for improving cumbersome, time-consuming, and labor-intensive traditional immunoassays.

Various substances, including nanoparticles [24], glass microbeads [25, 26] and magnetic microbeads [27, 28], have been used as carriers. In this study, we use carboxylic resin beads as an alternative support for the immobilization of coating-antigen and preparation of the immunoaffinity column. After being activated with N-(3-Dimethylaminopropyl)-N-ethyl- carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (N-hydroxy-2, 5-pyrrolidinedione, NHS), the carboxyl groups provided with good hydrophilicity [29] and binding sites for more coating-antigen immobilization, which will exhibit a lower LOD and wider range for the detection of clenbuterol.

To obtain a simple flow injection chemiluminescence method for the determination of CLB, we designed an immunoaffinity reactor based on a new type of carboxylic resin beads used as an immunosensor. To the best of our knowledge, it is the first time that the immunoaffinity column using resin beads has been used for the detection of CLB. This CL immunosensor is simple, rapid and sensitive for bioassays and shows acceptable precision. This approach will extend the application of immunoaffinity reactor by using carboxylic resin beads in immunoassays, and open new avenues for the detection of food additive residue in the future.

Experimental section

2.1 Reagents and materials

Horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (HRP-GaRIgG) was purchased from Zhongshan Gold Bridge biotechnology Co. Ltd (Beijing, China). Clenbuterol was obtained from Yabang Chemical Industry Co. Ltd (Changzhou, China). Bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma-Aldrich Co. Ltd (USA). Carboxylic resin beads (diameter: 150 μm; sphericity: >99%; degree of crosslinking: 7%; content of water: 30-40%) were obtained from Nanjing Microspheres Hi-Efficiency Isolation Carrier Co. Ltd. (Nanjing, China). Tris(hydroxymethyl)aminomethane was purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). EDC and NHS used as carboxyl group activator were purchased from Merck and Aldrich, respectively. Hydrogen peroxide (30%) was bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), and its working solution was prepared with 0.1M pH 8.5 Tris-HCl buffer solutions daily. A stock solution of luminol (0.01M) was prepared by dissolving 1.77g of luminol in 1000mL of 0.1M NaOH. *p*-Iodophenol (PIP) stock solution (0.01M) was prepared by dissolving 1.1g of PIP (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) in 50mL dimethylsulfoxide (DMSO) and then diluted with water to 500mL and kept in the dark. Prior to use, luminol and PIP stock solutions were mixed and diluted by using 0.1M pH 8.5 Tris-HCl buffer solutions. Antigen and antibody were prepared with 0.01M (pH 7.4) phosphate-buffered saline (PBS). All other regents were of analytical grade and used without further purification. All aqueous solutions were prepared with sub-boiling distilled deionized water.

Preparation of the clenbuterol antigen and clenbuterol antibody

The clenbuterol-protein conjugates were prepared as follows. Briefly, 5.00 μg mL^{-1} clenbuterol was dissolved in 600 μL of water, and the solution was adjusted to pH 1.5 with

1.00 M HCl, then 1.00 mL 10.00 mg mL^{-1} $NaNO_2$ was dripped into the clenbuterol solution. The mixture was agitated and reacted for 4 h at 4℃. Then 1.50 mL PBS containing 100.00 mg of BSA (or 66.00 mg of OVA) was added into the mixture. The obtained clenbuterol-protein conjugates were lyophilized and stored in the refrigerator at -20 □ after it was centrifuged and dialyzed against PBS buffer at 4℃ for 4 days. Finally the clenbuterol-OVA and clenbuterol-BSA were used as coating-antigen and immunogen, respectively.

According to the literature [30], four adult New Zealand rabbits were immunized with as-synthesized clenbuterol-BSA at 4-week intervals for four subsequent injections. The polyclonal antibody of clenbuterol (Ab1) was obtained from the antisera of animals, and stored at -60 □ until use. It should be mentioned that all experiments in live animals were performed in compliance with the relevant laws and institutional guidelines of China, and the institutional committees have approved the experiments.

2.2 Preparation of immunoaffinity column

The preparation of resin beads for immunoaffinity column was carried out as the following steps. The resin beads were swelled with 20% ethanol, and washed with 0.01 M 2-morpholinoethanesulfonic acid (pH 5.5) for three times. Then the suspension of beads was mixed with 30.00 mg EDC and 30.00 mg NHS for 2 h under constant stirring at room temperature. The resin beads were washed three times with 0.01 M PBS, and 5 μL of clenbuterol coating-antigen (20.00 μg mL^{-1}) was added into the resin beads. Then the suspension was allowed to stand for 24 h in refrigerator at 4℃. After that, the resulting coating-antigen immobilized resin beads were slowly washed with streams of 0.01 M pH 7.4 PBS and treated for another 4h with blocking buffer (PBS containing 1% bovine serum albumin). Finally, they were thoroughly washed with PBS (pH 7.4) and packed into a glass

tube (1.6mm i.d. and 4.0cm length) with the volume about 100 μL, which was stored in 0.01 M PBS at 4 ℃ until use.

2.3 Apparatus

All the chemiluminescence measurements were carried out with IFFM-E flow injection chemiluminescence analyzer (Remex Electronic Institute Limited Co., Xi'an, China) equipped with an IFFS-A multifunction chemiluminescence detector (Remex Electronic Institute Limited Co., Xi'an, China). A schematic diagram of the instrument is shown in Scheme 1. Two peristaltic pumps of the IFFM-E Luminescence Analyzer were used to deliver all solutions. An injection valve with a 100 μL loop was used to introduce the CL substrates. Teflon tubes (0.8 mm i.d.) were used to connect all the components in the flow system. The immunoaffinity column was positioned in front of the photomultiplier (PMT). The CL signal was detected by the PMT at −600 V with no wavelength discrimination and recorded with computer by employing the IFFM-E flow-injection CL analysis system software. The Scanning Electron Microscope (SEM) images of carboxylic resin beads were obtained with EVO18 scanning electron microscope (Zeiss, Germany).

Scheme 1.

2.4 Analytical procedure

The immunocomplex(CLB/Ab1), the mixture of 5 μL clenbuterol standard solutions and 5 μL 5.00 μg mL^{-1} clenbuterol antibody, was diluted with 0.01 M pH 7.4 PBS in a volume ratio of 1:800. 10 μL 6.00 μg mL^{-1} HRP-GaRIgG was also diluted with 0.01M pH 7.4 PBS in a volume ratio of 1:800. Firstly, the prepared CLB/Ab1 was introduced into the immunoaffinity column at the flow rate of 0.50 mL min^{-1}. It formed a competition between the clenbuterol standard in the incubation solution and the coating-antigen modified on the beads for limited binding sites of the antibody. After being washed with 0.01M pH 7.4 PBS,

enzyme tracer was passed through the column at a flow rate of 0.50 mL min^{-1}, which took a period of 16 min for the immobilized CLB-antibody to capture the secondary HRP-GaRIgG. To remove the unbound immunoreagents, the column was washed with PBS containing 0.05% Tween-20 (PBST, pH 7.0) at a flow rate of 2.00 mL min^{-1} for 1 min. Then 100 μL CL substrates (*p*-iodophenol-luminol-H_2O_2) were carried into the flow system. Afterwards, the CL signal was captured and recorded by the detector. Finally, the resin beads could be regenerated with 0.10 M pH 2.2 glycine-HCl for 1 min at a flow rate of 2.00 mL min^{-1}

As shown in Scheme 1, the coating antigen (clenbuterol-OVA) is immobilized on the surface of the carboxylic resin beads. The Ab1 against clenbuterol is bound at the sites to form the immunocomplex. Then HRP-GaRIgG is added and will bind to the Ab1. In the presence of *p*-iodophenol-luminol-H_2O_2, the horse radish peroxidase will catalyze the CL reaction and produce light emission. There is a competition between clenbuterol and the coating antigen to react with the limited binding sites of the primary antibody. Thus the decrease of CL intensity is proportional to the amount of clenbuterol standard in the solution.

Results and discussion

3.1 Characterization of carboxylic resin beads

The resin beads are carboxyl-activated polystyrene-divinylbenzene material. The carboxyl groups offer good hydrophilicity and binding sites for antigen immobilization after being activated with EDC and NHS. The SEM of resin beads demonstrates regular orbicular structure and smooth surface (Fig. 1a). While coating-antigen is bound to the carboxyl group, the surface morphology shows obviously changes (Fig. 1b), indicating the formation of coating-antigen immobilized resin beads.

Figure 1.

3.2 Kinetic characteristics of CL reaction

The kinetic characteristics of the proposed CL reaction were studied by a stop-flow injection method after the baseline was steadily recorded. CLB/Ab1 and HRP-GaRIgG (or PBS) were injected into reactor, respectively. The signal was produced after the injection of 100 μL substrate (*p*-iodophenol-luminol-H_2O_2) into the column. Fig. 2 shows the changes of CL intensity after flowing of different solutions through the column. As seen in Fig. 2, the weak CL signal of the p-iodophenol-luminol-H_2O_2 (curve a) is remarkably increased in the presence of HRP-GaRIgG (curve b). Furthermore, it is found that the rate of the reaction is so fast that the CL intensity reached the peak maximum only 2 s from reagent mixing, and it took about 15 s for the signal decline to the base line.

Figure 2.

3.3 Optimization of CL detection conditions

In order to achieve a highly sensitive immunoassay by the present method, various CL detection conditions such as flow rate, concentration of substrate were optimized to obtain the most suitable operational condition. According to the kinetic curve (shown in Fig. 2), the chemiluminescence is extremely rapid emission process. Therefore, a higher flow rate is necessary for the maximum collection of the emitted light in the flow cell. However, too high flow rate can affect the CL reaction and consume more substrates. It was found that the maximum CL intensity was obtained when P1 was the flow rate of 2.00 mL min^{-1} for this FIIA system. Since the sampling immobilized time must be sufficient, the flow rates of P2 were studied in the range of 0.10 to 2.00 mL min^{-1}. The CL signal is unchanged when the flow rates of P2 exceeded 0.50 mL min^{-1}. The considering the reagent consumption, 0.50 mL min^{-1} was chosen as the flow rate free antibody to on the bead. The effects of the concentration of CL substrates including luminol, PIP and H_2O_2 on CL intensity were also studied in detail. Their optimal concentrations are 0.70, 0.60and 3.80 mM, respectively. The

CL emission in Tris-HCl buffer solution was more stable than that in other buffers such as PBS, Na_2CO_3-$NaHCO_3$. Thus all the substrates were prepared in 0.10 M Tris-HCl buffer solution (pH 8.5).

3.4 Optimization of the immunoassay procedure

The key factors that affect the immunoassay procedure are the conditions of the immunoreagents and the incubation time of the mixture in the immunoaffinity column. Firstly, the working concentrations of the immunoreagents were investigated to give a compromise with good sensitivity and a wide assay range. 10 μL different concentrations of clenbuterol coating-antigen immobilized on resin beads were investigated for immunoaffinity column preparation. As shown in Fig. 3a, with the increasing concentrations of clenbuterol coating-antigen, CL signals increases and trends to a plateau at 20.00 μg mL^{-1}. Thus 20.00 μg mL^{-1} of clenbuterol coating-antigen was selected as the optimal concentration. Using the same method, the concentrations of clenbuterol antibody and enzyme tracer were also optimized. And the optimal concentrations are 5.00 μg mL^{-1} (Fig. 3b) and 6.00 μg mL^{-1} (Fig. 3c), respectively.

To obtain high analytical performance, the incubation time of CLB/Ab1 was tested in detail. As shown in Fig. 3d, it is found that the CL intensity increases with increasing incubation time followed by verging to leveling off at 18 to 22 min. Considering the analytical performance and further development of this method for high samples throughput, 20 min was used as the incubation time in this immunoassay.

Figure 3.

3.5 CL detection of clenbutrol

Under the optimum conditions, the CL intensity decreases with increasing of clenbutrol concentration (shown in Fig. 4) and allows the measurement of clenbuterol over the range of

0.40 – 120 ng mL^{-1}. The CL intensity decreases linearly response to the increasing of clenbuterol concentration, with a linear regression equation of *I* (relative units) = 7519.87-53.11C (ng mL^{-1}) (r=0.999, n=9), and the limit of detection is 0.20 ng mL^{-1} at S/N of 3. The analytical performance of this immunosensor is better than that of the other existing sensor [31, 32] in the detection of clenbuterol. Compared to our previously published works [33, 34], the proposed method eliminates the tedious electrode polishing and multilayer modifications, which become more simple and convenient for the detection. Moreover, the immunosensor could be regenerated, avoiding the waste of coating-antigen. Compared to the other reported CL methods (listed in Table 1), the proposed clenbutrol detection method possesses excellent performance such as wide linear range, low detection limits and high sensitivity.

Figure 4.

Table 1**.**

3.6 Reproducibility and specificity for the clenbutrol sensing process

The coating-antigen modified carboxylic resin beads are extremely stable, in which no obvious changes are observed after 5-month storage. Furthermore, the binding of CLB antibody and coating-antigen is reversible. Here we tested reproduction reagent of 0.10 M glycine-HCl (pH 2.2), CH_3OH-H_2O (1:1) and 1.00 M NaCl, respectively. Obviously, the most efficient dissociating reagent was found to be 0.10 M glycine-HCl (pH 2.2), which allowed a fast and complete dissociation of the immunocomplex with a regeneration efficiency of 92.5 %. To assesse the immunosensor regeneration aptitude, a series of control experiments were conducted for deceting of 40.00 ng mL^{-1} CLB. The strong signal could be detected by flowing of CLB/AB1 and HRP-GaRIgG into a new assembled immunosensor (Fig. 5a), while the CL intensity extremely decreased after washing with glycine (Fig. 5b). Then injection the

same immunoassay solution into the regenerative immunosensor, 96.32% CL signal (Fig. 5c) is observed compared to the primary signal (Fig. 5a). All these results demonstrated a complete detachment of the firstly captured antibody exists after a regeneration cycle. After 20 times of regeneration, there was no observable loss of immunoactivity in columns. The reproducibility of columns shows an acceptable reproducibility and can be used repeatedly. As shown in Fig. 5, the relative standard deviation (RSD) of three states CL responses is 0.69%, 1.08%, 0.87%, respectively (n=11). The coincident signals indicate the excellent reliability and stability of the immunosensor, which can be applied to the CL detection.

Figure 5.

The RSD of CL signals at 40.00 ng mL^{-1} clenbuterol in the incubation solution with five immunosensors fabricated independently is 5.23%, giving an acceptable fabrication reproducibility of the immunosensors.

In order to evaluate the specificity of the CL immunosensor, we selected some other β-agonists including salbutamol, phenylethanolamine A and ractopamine as the interfering species since they might exist in some real samples. Under the optimum conditions, the results of the tested concentrations at 10.00 ng mL^{-1} for these agonists are shown in Table 2. Owing to their very analogous chemical structures, salbutamol shows the cross-reactivity about 37.20 % with the CLB antibody. Phenylethanola- mine A and ractopamine do not cause the interference in the test, which suggests that this immunosensor has an acceptable specificity for the determination of CLB in real samples.

Table 2.

3.7 Real sample analysis

To evaluate its potential application, the proposed method was applied to the determination of CLB in real samples including pork meat and liver. 10.00g of pork (or liver)

collected randomly from the market in Suzhou was taken for analysis. Extraction was performed with 60.00 mL 0.02 M HCl and kept at room temperature overnight. After the removal of precipitates, the supernatant from pork meat and liver samples was directly analysed. There were no detectable clenbuterol residues in all collected samples, thus they could be used as blank samples. To evaluate the reliability of the prepared immunosensor, HPLC was established for the detection of CLB in the real samples. Table 3 shows the samples of pork meat and liver spiked with CLB standards at different concentrations and measured by immunsensors and HPLC method. It confirmed that there are no significant differences for the detection of CLB between this method and HPLC. As shown in Table 3, the recoveries from the samples are excellent, and vary from 91.5% to 104.0%, which further proves the applicability of the immunsensor for analysis of clenbuterol in real samples.

Table 3.

Conclusions

The paper presents an immuno-chemiluminescent method for the detection of clenbuterol in meat products. This is important as clenbuterol has been used by unscrupulous producers to increase the yield of muscle meat over fat in life stock, leading to adrenergic effects in unsuspecting consumers. The carboxyl-activated resin beads show good immobilization capacity to coating-antigen and improve the luminescence signal efficiently. The resulting immunosensor possesses the advantages of low-cost, good storage stability and fabrication reproducibility. This proposed method has been used in the detection of real samples with satisfactory results, which could be a promising perspective method for other small molecular compounds and extend the application of CL immunosensing.

Acknowledgments

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Table 1. Comparison of limit of detection and linear range of proposed method with other published methods.

LOD	Linear range	Ref.
0.03 ng mL^{-1}	0.10-5.00 ng mL^{-1}	[35]
0.50 ng mL^{-1}	1.00-500.00 ng mL^{-1}	[36]
1.20 nmol l^{-1}	5.00-40.00 nmol l^{-1}	[6]
0.20 ng mL^{-1}	0.40-120.00 ng mL^{-1}	This work

Table 2. Cross-reactivity between CLB antibody and the interfering β_2-agonists at 10.00 ng mL^{-1} (n = 3).

Compound	Analysis result (ng mL^{-1})	RSD (%)	Cross-reaction (%)
Salbutamol	3.72	2.68	37.20
PhenylethanolamineA	Not found	-	-
Ractopamine	0.60	4.73	6.00

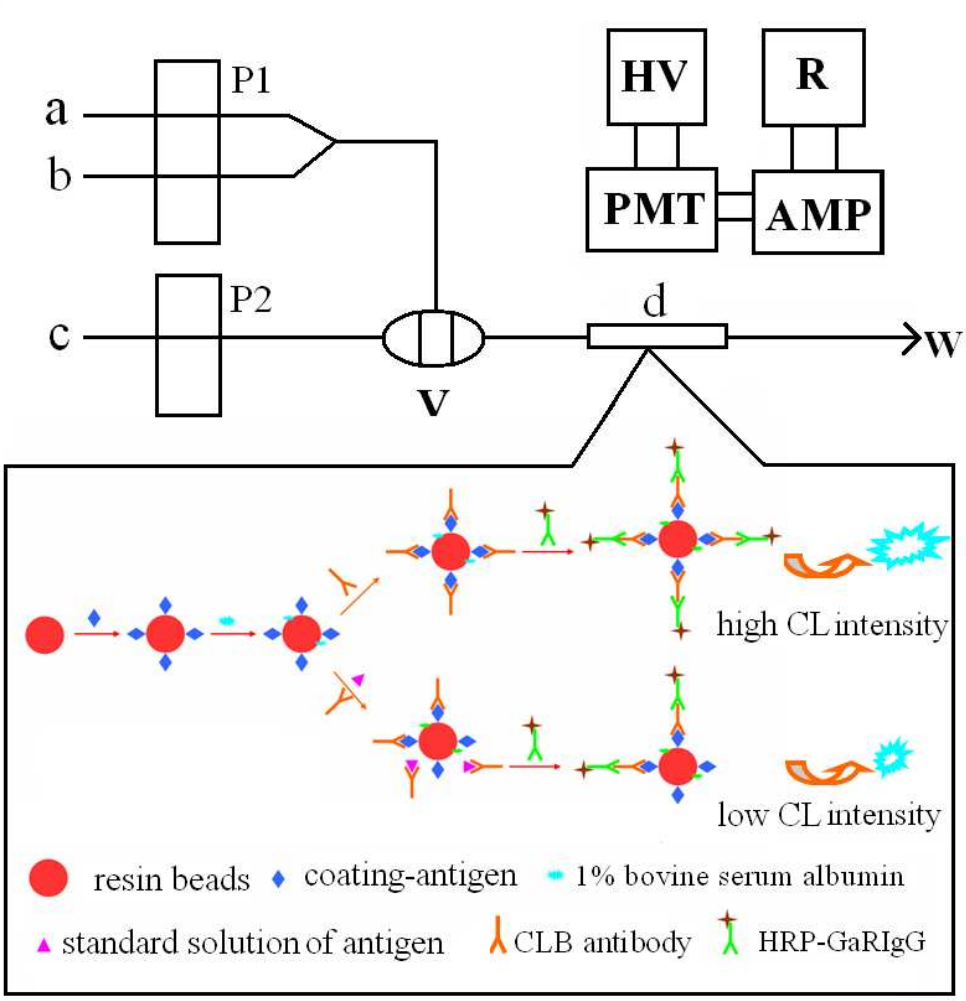
Table 3. Recovery tests of CLB spiked in the real samples by the proposed method and HPLC (n = 3).

Samples	Added (ng mL^{-1})	[a]Found (ng mL^{-1})	[a]Recovery (%)	[b]Found (ng mL^{-1})	[b]Recovery (%)
Pork No.1	10.0	10.4	104.0	10.8	108.0
Pork No.2	20.0	18.7	93.5	19.4	97.0
Pork No.3	40.0	40.8	102.0	41.3	103.2
Pork liver No.1	10.0	9.7	97.0	10.5	105.0
Pork liver No.2	20.0	18.3	91.5	20.7	103.5
Pork liver No.3	40.0	41.2	103.0	42.4	106.0

[a] Determination by the proposed method
[b] Determination by HPLC

Figure captions

Scheme 1.



Scheme 1. Schematic diagram of the FIIA system manifold: (P1) peristaltic pump; (P2) syringe pump; (a) luminol and PIP; (b) H_2O_2; (c) resin beads, sample, antibodies, washing buffer; (V) injection valve; (d) immunosensor; (PMT) photomultiplier; (HV) negative high voltage supply; (AMP) amplifier; (R) recorder; (W) waste.

Figure 1.

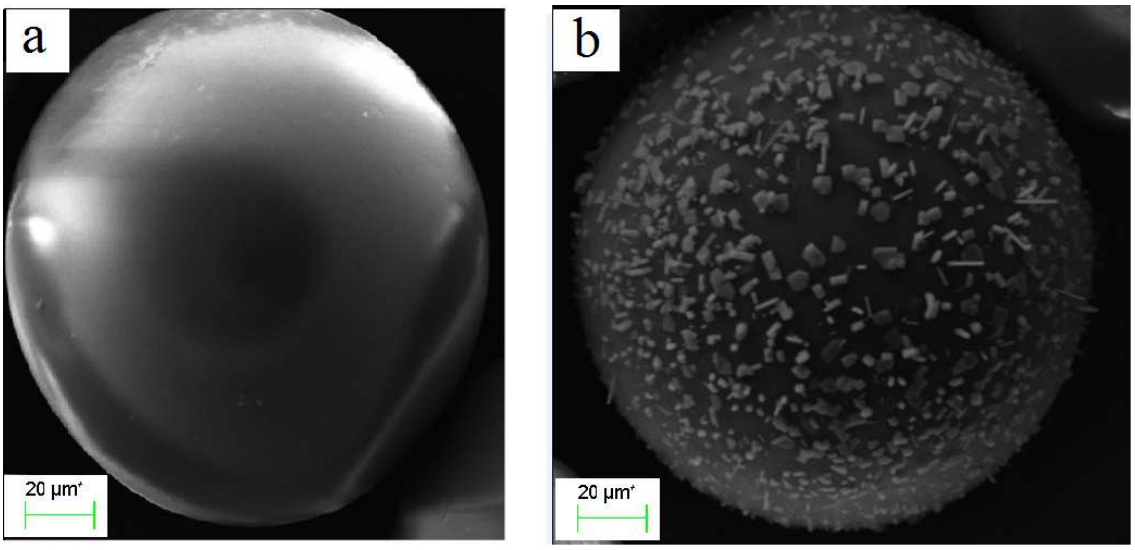


Figure 1. SEM images of (a) naked carboxylic resin beads and (b) clenbuterol coating-antigen immobilized resin beads.

Figure 2.

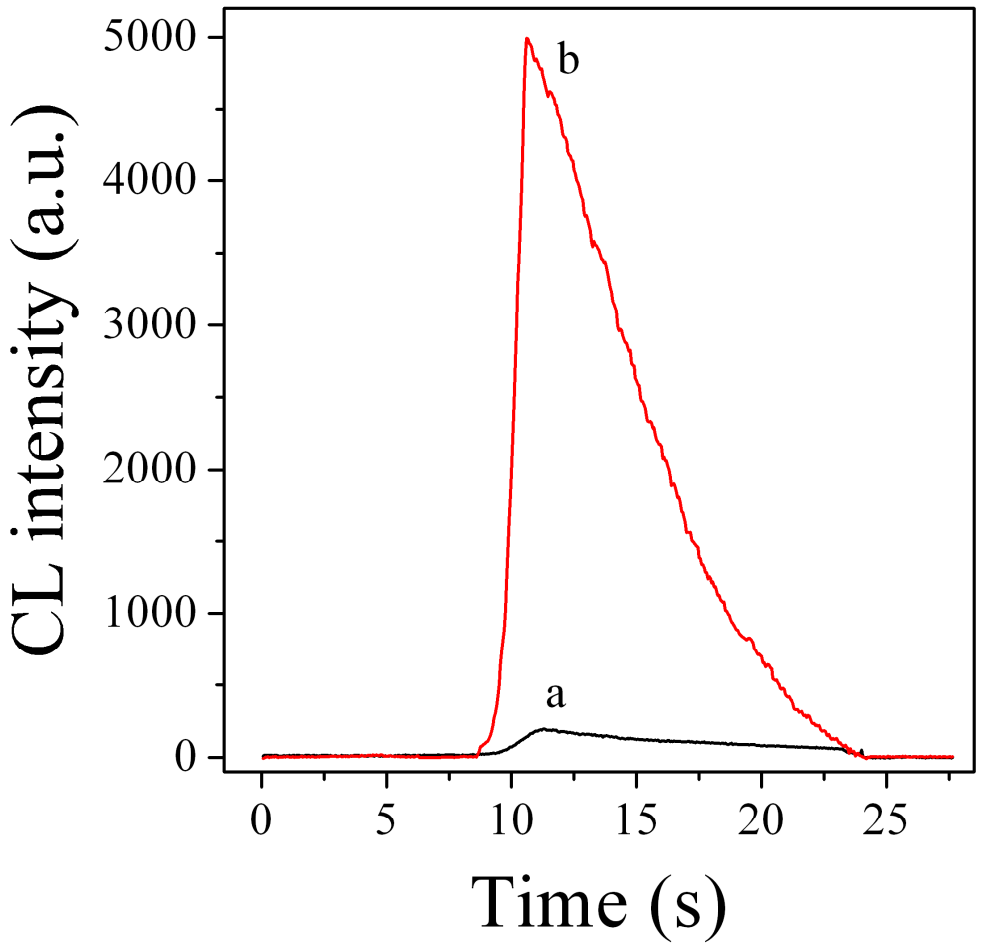


Figure 2. Kinetic curve of luminol-PIP-H_2O_2 CL system: PBS (pH 7.4) in the absence (a) and presence of the 10.00 μL 6.00 μg mL^{-1} of HRP-labeled goat anti-rabbit IgG (b), respectively. Conditions: 100.00 μL of Tris-HCl (pH 8.5) containing 3.80 mM H_2O_2, 0.70 mM luminol and 0.60 mM PIP was injected.

Figure 3.

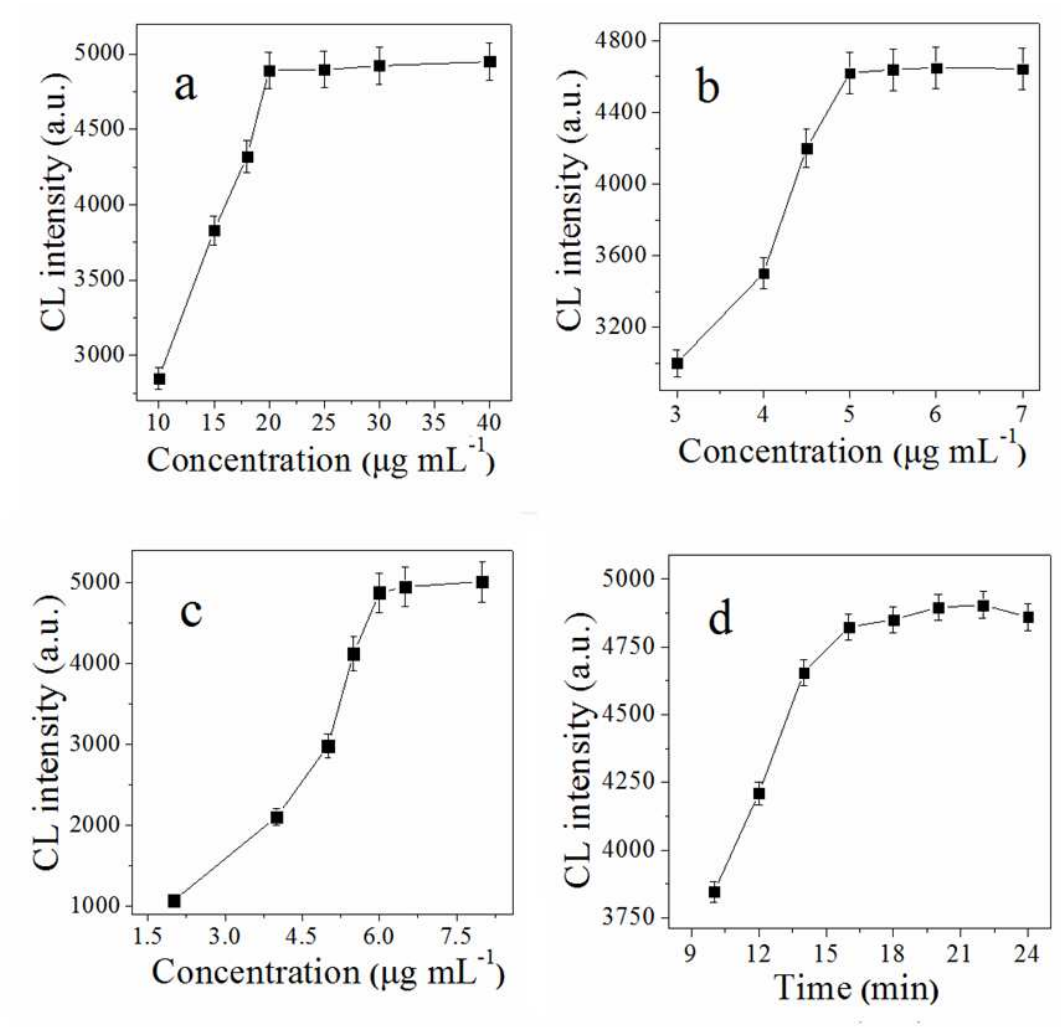


Figure 3. Effects of concentration of clenbuterol coating-antigen (a), clenbuterol antibody (b) and HRP-GaRIgG (c), and (d) pre-incubation time of clenbuterol and clenbuterol antibody on CL intensity.

Figure 4.

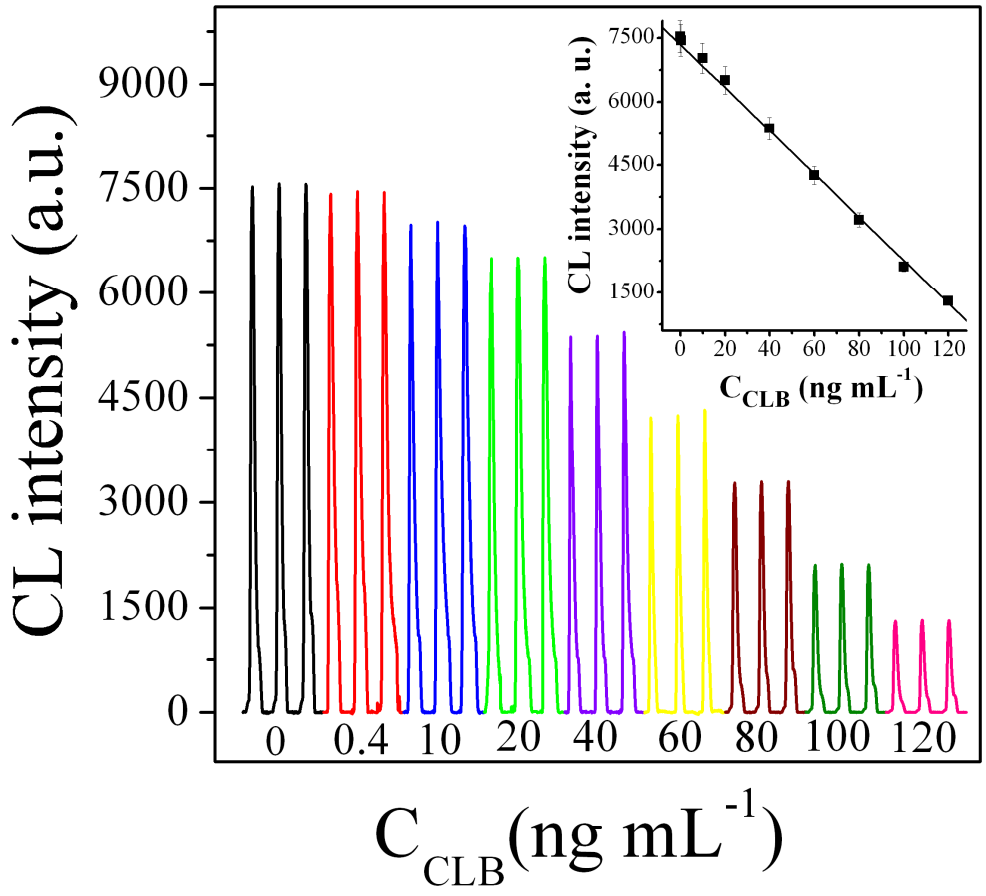


Figure 4. The CL responses of the immunosensor for detecting different concentrations of clenbuterol. Inset: calibration curve, where n = 5 for each point. All the tests were performed under the optimal conditions.

Figure 5.

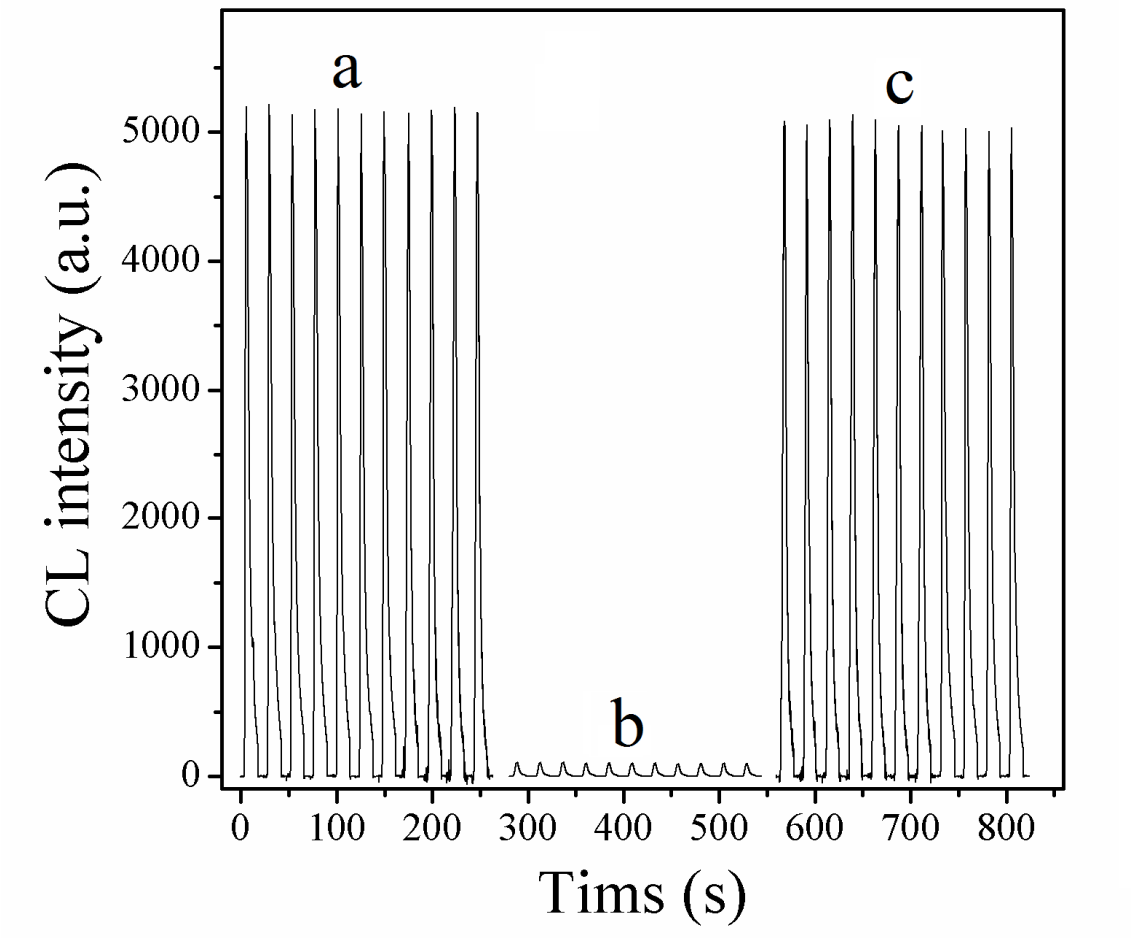


Figure 5. CL intensity of (a) a new assembled immunosensor flowing with CLB/Ab1+ HRP-GaRIgG, (b) the immunosenser washing with 0.1 M glycine-HCl (pH 2.2) and (c) the regenerative immunosensor injected into CLB/Ab1+ HRP-GaRIgG.