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Clenbuterol Assay by Spectral Imaging Surface Plasmon Resonance Biosensor System

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Abstract To prevent illegal use of clenbuterol and for quality control in the food industry, more efficient and reliable methods for clenbuterol detection are needed. In this study, clenbuterol was detected using a spectral imaging surface plasmon resonance sensor system via two inhibition methods: (1) the target site compensation method, in which anti-clenbuterol antibody was immobilized on the sensor chip as a bioprobe and (2) the solution competition method in which a clenbuterol-BSA conjugate was immobilized on the sensor chip as the bioprobe. The detectable clenbuterol concentration ranged between 6.25 and 100 μg/mL for both methods. The clenbuterol limit of detection for the target site compensation method and solution competition method are estimated to be 6.7 and 4.5 μg/mL, respectively. The proposed methods were successfully applied to the detection of clenbuterol molecules and were found to have high specificity and high-throughput and were label free and operationally convenient.

Keywords Surface plasmon resonance biosensor · Low-molecular-weight hapten detection · Multichannel measurement · Food safety

Abbreviations

CLEN	Clenbuterol
anti-CLEN antibody	Anti-clenbuterol polyclonal antibody
PDMS	Polydimethylsiloxane

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Introduction

Clenbuterol (CLEN) is a β-adrenergic agonist [1]. The earliest use of clenbuterol was in the treatment of human and animal respiratory conditions. Later, it was found that CLEN has an impact on nutrient partitioning and can lead to a greater lean meat production in livestock by transforming nutrients into muscle instead of adipose tissue [2]. However, significant side effects cannot be ignored. When it is used illegally as an additive in animal feed, the normal growth of livestock is hampered. In addition, CLEN is a very stable chemical and does not decompose, even at high temperatures [3]. Due to its high stability, CLEN residues in the meat and viscera of livestock are not eliminated by cooking before eating. Serious cardiovascular and central nervous system disorders may occur when humans take CLEN in high amounts. In addition, long-term consumption of meat with residual CLEN may result in cancer and ultimately death [4, 5]. In order to improve the quality of meat and protect the interests of consumers, many countries have established relevant laws forbidding the use of CLEN in stockbreeding.

Various methods for detecting CLEN have been developed to ensure food safety. At present, the primary technologies used to identify CLEN include enzyme-linked immunosorbent assay (ELISA), gas chromatography–mass spectrometry (GC-MS), and high-performance liquid chromatography (HPLC) [6–8]. Though these methods have the advantage of sensitive detection and are well-established experimental techniques, they are also time consuming, require complex operations, or have to be equipped with expensive instrument. In contrast from the methods above, a surface plasma resonance (SPR)-based system has many advantages, including the low cost of instruments, convenient operation, and real-time detection. Another appealing aspect of SPR is that analytes are detected without labeling, which saves time and does not damage the bioprobes [9–11].

In 1902, R.W. Wood first reported the surface plasmon resonance phenomenon [12]. Later, SPR technology was used for gas sensing and biosensing by Nylander and Liedberg [13, 14]. Today, SPR measurement has become a mature biosensing technology for detecting biomolecular interactions [15]. SPR applications are steadily increasing for disease diagnosis, drug screening, environmental monitoring, food safety monitoring, and so on [16–20]. However, most SPR biosensors only provide a single sample measurement, which is time consuming and of low efficiency. Previously, we developed and reported a spectral imaging surface plasmon resonance (spectral SPRi) biosensor system [21, 22] for multichannel measurement. In this work, we have applied the SPRi for CLEN detection.

Materials and Methods

Chemicals and Reagents

Clenbuterol hydrochloride (CLEN) was purchased from Beijing Aoke Biotechnology Co., Ltd. Anti-clenbuterol polyclonal antibody (anti-CLEN antibody) was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. 11-Mercaptoundecanoic acid (MUA) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from J&K Chemical Technology Co., Ltd. *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Chemical Company (St. Louis, USA). Ethanolamine solution and other chemicals of analytical grade were purchased from standard commercial sources.

Methods

Instrumental Method

The details of the spectral SPRi instrument have been reported previously [21, 22]. Briefly, with the incident angle fixed, we synchronously obtained SPR spectrum curves for each Au spot on the array using scanning wavelength in the detection procedure. Different analytes captured in the sensing surface spot induce changes of resonance dip in the spectrum, which can then be identified accordingly. In our SPR system, the sensing surface is composed of 12 spots arrays, which makes it possible to analyze large numbers of biomaterials synchronously in one sensor chip. Thus, detection of many CLEN samples can be performed at one time, bypassing a traditional time-consuming step. Besides, our SPRi biosensor is based on wavelength modulation. Compared with traditional SPRI biosensors based on light intensity modulation, our biosensor is more stable in terms of ambient light.

Preparation of CLEN–Bovine Serum Albumin (CLEN-BSA) Conjugate with Diazotization Method

CLEN-BSA was made as follows [2]:

Five milligrams of reagent-grade clenbuterol hydrochloride was dissolved in 1 mL of pre-cooled 1 M HCl, while the pH was adjusted to 0.5 by adding 500 μL deionized water. To this mixture, 60 μL of 0.2 M $NaNO_2$ (3×20 μL, with an interval of 20 s) was added slowly while stirring at a low speed at 4 °C. After stirring for 1 h, the reaction was tested for completion using a potassium iodide-starch test paper (that the color of the test paper turned to dark purple indicated the reaction was completed).

BSA (30 mg) was dissolved in 1 mL of 0.1 M carbonate buffer, pH 9.5. Then, the diazo derivative prepared above was added dropwise into the BSA solution. The pH was kept between 9.0 and 9.5 by adding 1 M NaOH. This solution was kept at 4 °C while stirring in the dark for about 3 h. Dialysis was performed with PBS solution (0.01 M, pH 7.2~7.4) for 2 days. The conjugating reaction was monitored with UV-visible absorption spectrum. Dialysis was performed until the absorption spectrum of the ultimate replaced dialysate (containing the excess clenbuterol) approximately equaled the baseline. The final CLEN-BSA was stored at −20 °C until needed.

Preparation of the Sensor Chip

The BK7 glass slides have the same refractive index as the prism of the SPR biosensor. These sensor chips, which measure 30×25×2 mm, were sonicated in deionized water, acetone, and ethanol, sequentially, for 10 min by ultrasonic washing. The chips were then rinsed by deionized water and blown dry with nitrogen.

A vacuum sputtering apparatus was used to coat the chips with 3×4 spot arrays of 2 nm-thick Cr film and 50 nm-thick Au film (diameter of each spot: 1 mm). Then, the freshly fabricated chip was immersed overnight in a solution of MUA (5 %, *v*/*v*) in ethanol at room temperature to form a self-assembled monolayer over the gold spot arrays.

After the self-assembly step, the sensor chips were rinsed with ethanol and deionized water sequentially and blown dry with nitrogen. Then, the chips were masked with a polydimethylsiloxane (PDMS) holey cell, ensuring each spot exactly in a PDMS cell (10 μL). For convenience, the spots were named as shown in Fig. 1.

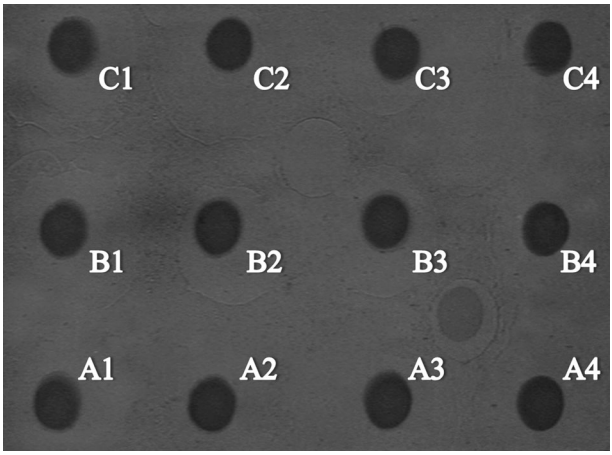

Fig. 1 Surface plasmon resonance (SPR) image of the 3×4 array sensor chip

Target Site Compensation Method

After the PDMS holey cell was masked on the sensor chip, a mixture of 0.1 M sulfo-NHS and 0.4 M EDC solution (1:1 *v*/*v*) was dropped by a pipette in each PDMS cell and maintained at 37 °C for 30 min to activate the carboxyl groups on the chip surface. The chip was then washed with deionized water, and anti-CLEN antibody solution (30 μg/mL) was pipetted into each PDMS cell and incubated on the surface of Au film at 37 °C for 1 h. This incubation allowed bond formation between the amine group of the anti-CLEN antibody and the activated carboxylic acid group on the chip surface. Next, the chips were rinsed exhaustively with PBS (pH 7.4) and the residual activated carboxyl groups were then blocked with 1.0 M ethanolamine (pH 8.5) solution to limit non-specific absorption. After incubating for 30 min at 25 °C, the Au spots were rinsed with PBS and deionized water sequentially.

The subsequent manipulation processes on the Au spots of sensor chip are graphically shown in Fig. 2, using the spot treatments as follows:

A1–A4: These spots were immersed in CLEN solutions of different concentrations (B1 ~ 6.25 μg/mL; B2 ~ 12.5 μg/mL; B3 ~ 25 μg/mL; B4 ~ 50 μg/mL) and incubated at 37 °C for

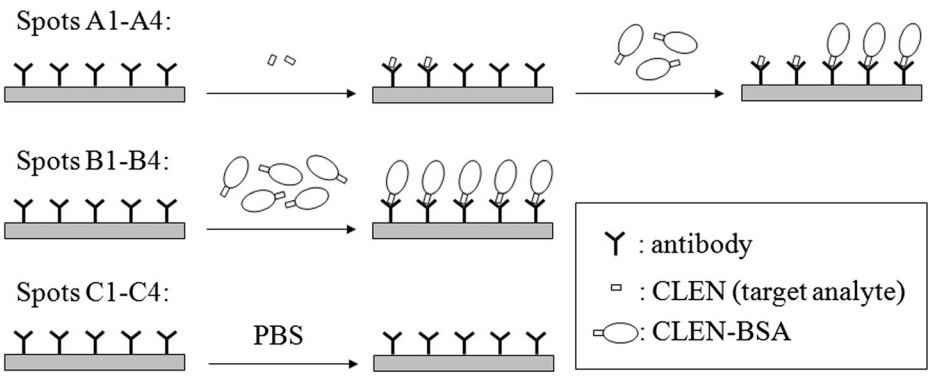

Fig. 2 Schematic diagram of target site compensation method

1 h. This was followed by an exhaustive PBS rinse and then an excess concentration of CLEN-BSA solution was dropped in these reaction areas.
B1–B4: These spots were immersed in 35 μg/mL CLEN-BSA solution only.
C1–C4: These spots were not treated with any additional reagents, except PBS was added to prevent evaporation. They were used as the reference to calculate the resonance wavelength shift of other spots.

Finally, all spots were washed with PBS and deionized water in sequence, and then the prepared sensor chips were analyzed with the spectral SPRi biosensor.

Solution Competition Method

In this method, all steps before the EDC/sulfo-NHS activation procedure were performed as described in "Target Site Compensation Method" section. After the carboxyl groups were activated using EDC/sulfo-NHS, the CLEN-BSA was immobilized on the Au surface, followed by an exhaustive rinse with PBS. Then after blocking the residual carboxyl groups with the ethanolamine solution, the Au spots were rinsed with PBS and deionized water sequentially.

The subsequent manipulation processes of this method are graphically shown in Fig. 3, using the spot treatments as follows:

A1–A4: An anti-CLEN antibody solution (25 μg/mL) was applied to these spots.
B1–B4: The mixture of fixed concentration anti-CLEN antibody (25 μg/mL) and series of CLEN concentrations (B1~6.25 μg/mL; B2~12.5 μg/mL; B3~25 μg/mL; B4~50 μg/mL) was dropped in these reaction areas (the CLEN and anti-CLEN antibody solutions were mixed and incubated at 37 °C for 30 min in advance).
C1–C4: There were no additional reagents added to these spots, except a PBS solution to prevent evaporation. They were used as a reference to calculate the resonance wavelength shift of other spots.

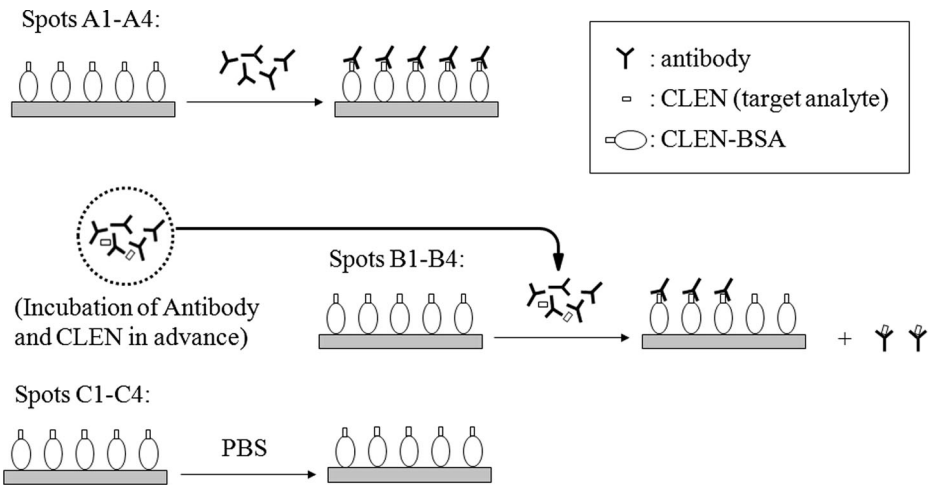

Fig. 3 Schematic diagram of solution competition method

After incubation and exhaustive rinsing with PBS, the array sensor chip was used for spectral SPRi measurement.

Results and Discussion

Principle of Inhibition Methods

Generally, it is difficult to detect low-molecular-weight analytes (<2000 Da) via direct methods by current SPR technology [23]. That is because CLEN only induces a weak SPR response when binding to the sensing surface, due to a small molecular weight (only 277.19 Da) [24]. So two competition methods were applied here for CLEN detection.

In inhibition methods, CLEN must be conjugated with a large carrier protein molecule such as BSA to form a conjugate antigen (CLEN-BSA). CLEN-BSA was utilized to specifically bind to antibody, which can be quantitatively determinated by SPR instrument, and the result of it reflected the amount of analyte (CLEN). More was discussed in "Detection of CLEN with the Target Site Compensation Method" and "Detection of CLEN with the Solution Competition Method" sections.

Surface-Modifying Procedures Monitored by SPR Measurement

The surface modification processes such as MUA self-assembly, EDC/sulfo-NHS activation, and protein immobilization were monitored using the spectral SPRi method. Each step resulted in a shift of the SPR response wavelength dip, and therefore, the reliability of the modification processes was confirmed. In Fig. 4a, the three spectral curves correspond to each step of the surface modification process. We found that the resonance wavelengths became larger as additional modification reactions were performed on the sensor chip. The resonance wavelength shift of curve (2) relative to curve (1) indicates that carboxyl groups on the sensor chip were activated. The resonance wavelength shift of curve (3) relative to curve (2) indicates that the anti-CLEN antibody was well immobilized on the sensor chip. Likewise, in Fig. 4b, the resonance wavelength shift of curve (2) relative to curve (1) indicates that carboxyl groups on the sensor chip were activated. The resonance wavelength shift of curve (3) relative to (2) indicates that CLEN-BSA was immobilized well on the sensor chip.

Detection of CLEN with the Target Site Compensation Method

The target site compensation method was utilized for detection, as described in "Target Site Compensation Method" section and graphically shown in Fig. 2.

In "Target Site Compensation Method" section, spots were grouped in terms of concentrations (6.25, 12.5, 25, and 50 μg/mL, respectively). Here, we choose one group of spots containing C3, A3, and B3 for discussion. In this group, 25 μg/mL of CLEN was analyzed. In the detection period, for B3, all the binding sites of immobilized antibody were occupied by CLEN-BSA, causing a large SPR response. By contrast, as for A3, after CLEN inactivated part of binding sites of immobilized antibody, there would be less CLEN-BSA binding to the sensing surface, which lowered the SPR response.

Figure 5 shows the SPR spectral curves of spots C3, A3, and B3. The resonance wavelength shift ($\delta\lambda$) of A3 relative to C3 is the result of specific binding of CLEN-BSA on the

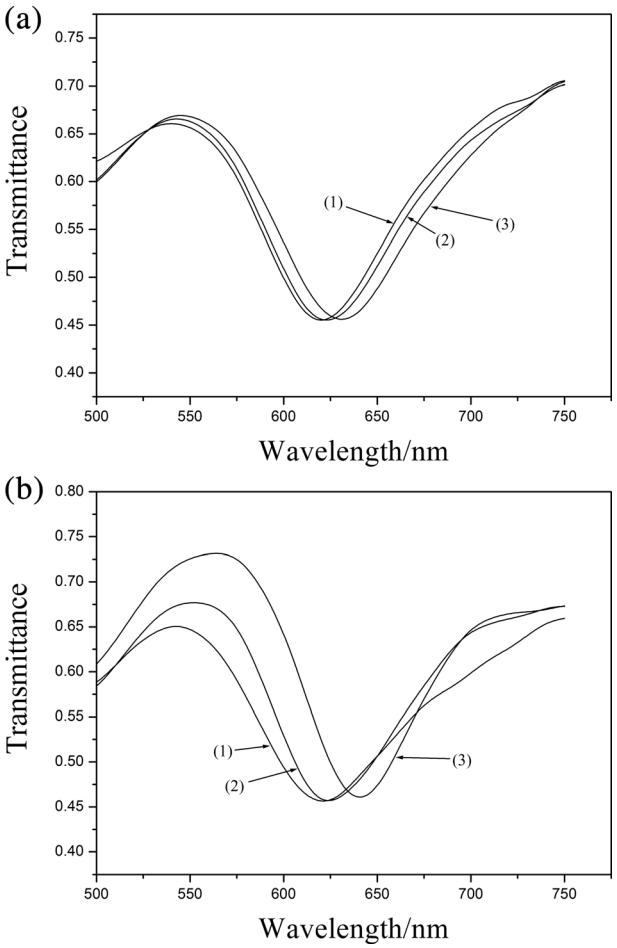

Fig. 4 **a** SPR spectral curves of modification steps on the biochip using the target site complementation method: (*1*) MUA self-assembled, (*2*) EDC/sulfo-NHS activation, and (*3*) immobilization of anti-clenbuterol polyclonal antibody (anti-CLEN antibody). **b** SPR spectral curves of modification steps on the biochip using the solution competition method: (*1*) MUA self-assembled, (*2*) EDC/sulfo-NHS activation, and (*3*) immobilization of CLEN-BSA

surface of spot A3. This shift depends on the concentration of the CLEN previously pipetted onto the surface of the spot.

Detection of CLEN with the Solution Competition Method

The solution competition method was described in "Solution Competition Method" section and graphically shown in Fig. 3. In "Solution Competition Method" section, spots were also grouped in terms of concentrations (6.25, 12.5, 25, and 50 μg/mL, respectively). Here, we choose one group of spots containing C4, A4, and B4 for discussion. In this group, 50 μg/mL of CLEN was analyzed. In this method, we mixed various concentrations of CLEN with a fixed concentration of anti-CLEN antibody in advance. Part of the anti-CLEN antibodies reacted with CLEN and was inactivated. This method utilizes immobilized CLEN-BSA as a probe to capture remnant dissociative anti-CLEN antibodies in the mixture, which indirectly reflects the amount of CLEN analyte.

Figure 6 shows the SPR spectral curves of spots C4, B4, and A4. The resonance wavelength shift of curve A4 relative to C4 is approximately 12 nm while $\delta\lambda$ of curve B4 relative to

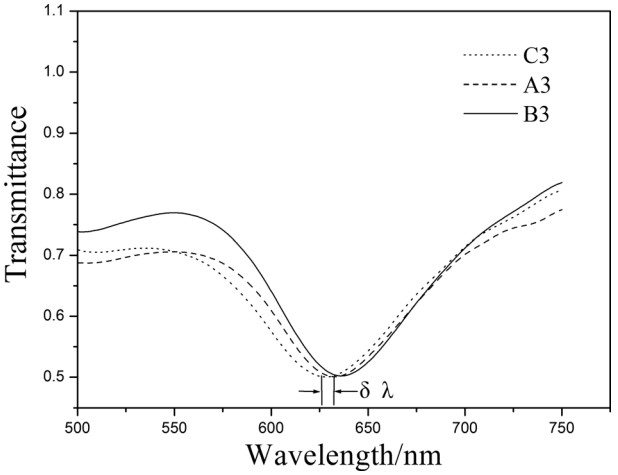

Fig. 5 SPR spectral curves of spots A3, B3, and C3; $\delta\lambda$ is the shift of A3 relative to C3, which can be used to characterize the specific binding between anti-CLEN antibodies and CLEN-BSA with CLEN interference

C4 is approximately 5 nm, which indicates the specific affinity reaction of CLEN with anti-CLEN antibody in the mixture. Thus, $\delta\lambda$ of curve B4 relative to C4 can be used to quantitate the concentration of CLEN in the mixture.

Comparison Between the Two Methods

Figure 7 shows the resonance wavelength shifts ($\delta\lambda$) in the SPR spectrum versus the concentrations of CLEN for the two methods tested. In both methods, the wavelength shifts decrease as the CLEN concentration increases. For the target site compensation method, we hypothesize that this is because the number of binding sites in antibody probes that remain available for CLEN-BSA binding dwindles as the number of CLEN molecules increases. As the concentration of CLEN increases, fewer CLEN-BSA, which cause the SPR response, are bound to the chip surface. For the solution competition method, the CLEN in the mixture competes with the immobilized CLEN-BSA for antibody binding. As the concentration of CLEN increases, more of the antibodies are bound, leading to fewer unbound antibodies in the

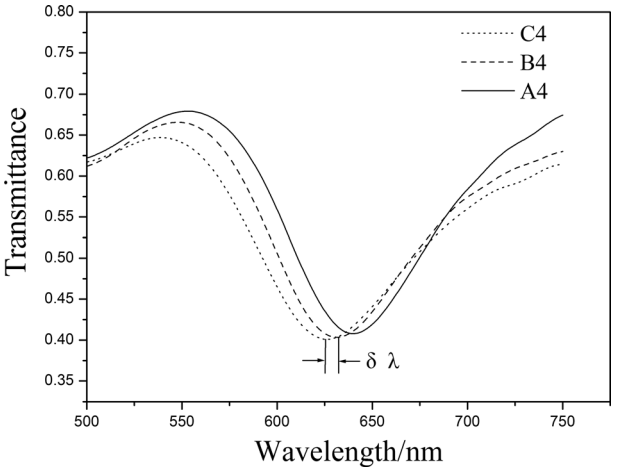

Fig. 6 SPR spectral curves of spots A4, B4, and C4; $\delta\lambda$ is the shift of B4 relative to C4, which can be used to characterize specific binding between CLEN-BSA and antibodies inhibited by CLEN

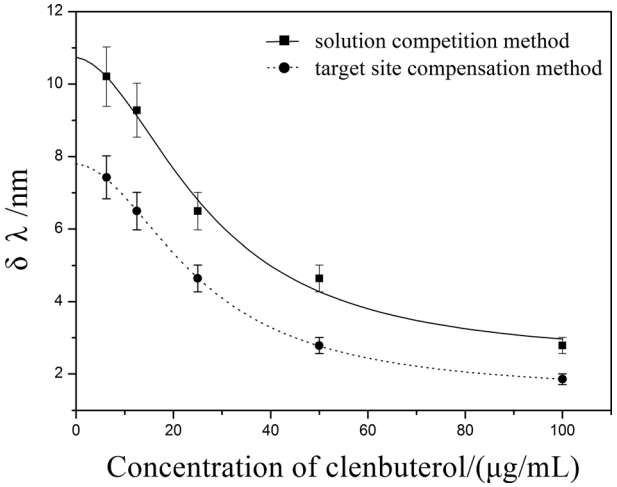

Fig. 7 Relationship between resonance wavelength shift and concentration of CLEN for each of the two methods

mixture and resulting in a decrease of SPR resonance wavelength shift. Comparing the two concentration curves in Fig. 7, it is clear that the solution competition method curve has a higher slope than the target site compensation method. We infer that this result indicates the solution competition method yields higher sensitivity in CLEN detection.

Both curves in Fig. 7 show an approximately linear relationship between the SPR wavelength shift and the CLEN concentration in the range 6.25–50 μg/mL. From the curves in Fig. 7, we estimate that the limit of detection (LOD) for the target site compensation method is 6.7 and 4.5 μg/mL for the solution competition method in the above concentration range. This is most likely because the molecular weight of the anti-CLEN antibody is much larger than that of the CLEN-BSA, which leads to a difference in SPR response. Moreover, it is estimated that the immune-binding property of anti-CLEN antibodies becomes weaker when it is immobilized on the surface, leading to smaller SPR resonance in the target site compensation method. In other words, the affinity constant between the anti-CLEN antibody and CLEN-BSA decreases when the antibody is immobilized on the Au surface via an amido bond [18]. Considering that the immune reactive ability of an antibody in solution is greater than those immobilized on the chip surface, we conclude that the solution competition method is more suitable for CLEN detection.

The LOD determined in this study is much higher than the one reported previously [25], which was obtained using BIACORE 2000. For the liquid flow system of Johansson and Hellenas, a large amount of clenbuterol liquid sample is injected for specific affinity binding. In our sensor chip, however, the required liquid sample is a small drop (10 μL), due to the restriction of the PDMS cell on the sensor chip. Therefore, the sample concentration generally needs to be larger than that of the liquid flow system. Despite this fact, our biosensor was utilized for these multichannel parallel measurements, which means it is suitable for swift screening of large numbers of samples.

In addition, from Fig. 7, we found that the resonance wavelength shifts still exist when the concentration of CLEN is very high. In this situation, all the anti-CLEN antibodies were inactivated and no specific affinity binding should occur on the surface. However, SPR resonance is still generated from non-specific absorption. This indicates that the final rinse step cannot absolutely eliminate non-specific absorption. This is an inevitable problem and a common difficulty in SPR measurement.

Conclusions

In summary, we have used the spectral SPRi system to detect clenbuterol with two methods. For the target site compensation method, anti-CLEN antibody was immobilized on the sensor chip, and then CLEN solution was pipetted and incubated. Finally, CLEN-BSA bonded specifically to the residual sites on the anti-CLEN antibody was added. For the solution competition method, CLEN-BSA was immobilized on the sensor chip, while a mixture of CLEN and antibody were incubated together and allowed to bind. Finally, this mixture was dropped and incubated on the chip surface. Our results demonstrate that both methods are capable of detecting CLEN, while the solution competition method is more sensitive than the target site compensation method. The solution competition method is more practical in use considering that no antibody immobilization step was necessary. The limit of detection (LOD) of the target site compensation method and solution competition method is 6.7 and 4.5 μg/mL, respectively. In general, LOD depends on many factors such as instrument sensitivity and stability, antibody quality, and experimental procedure. Although the LODs determined in this study are higher than previously reported [25], with advantages of multi-channel measurement, ease-of-use, and label-free method, our biosensor is believed to have a potential use in screening large numbers of samples in food testing.

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