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Surface Plasmon Resonance Sensor for Supersensitive Detection of Clenbuterol Using Molecularly Imprinted Film

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The supersensitive detection of clenbuterol was achieved by a method of combining molecularly imprinted polymer with surface plasmon resonance in this article. The detection limit of clenbuterol was 1×10^{-15} mol L^{-1}, and the changes in resonance angle ($\Delta\theta$) had a good linear relationship with the logarithm of clenbuterol concentration in the range from 1×10^{-15}–1×10^{-7} mol L^{-1}. Selectivity experiment showed that no $\Delta\theta$ was observed in response to salbutamol until the concentration of 1×10^{-5} mol L^{-1}.

Clenbuterol is a member of the artificial β-agonists family, which is used for treatment of asthma both in humans and animals.[1] It is also extensively misused in many countries of the world as a growth promoter in breeding livestock, which leads to a considerable decrease in fat deposits and increase in protein synthesis.[2] The clenbuterol residues in meat products have seriously threatened human health and related food trade.[3] In order to restrain this abuse, many countries have established tolerance levels of clenbuterol residues. For example, the maximum residue limits in animal tissue or urine is 0.5 ng g^{-1} (1.8×10^{-9} mol L^{-1}) in Europe and 1 ng g^{-1} (3.6×10^{-9} mol L^{-1}) in China.

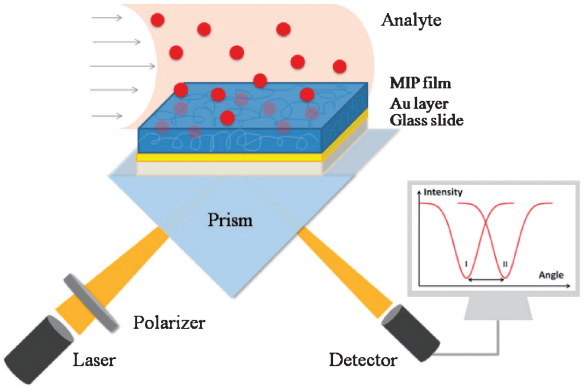
A number of analytical methods have been developed to determine clenbuterol including HPLC,[4] LC-UV,[5] LC-MS,[6] GC-MS,[7] ELISA,[8] and electrochemistry.[9,10] Even though several of these methods achieve low levels of detection, in practice they not only require complicated sample clean-up procedures and expensive instruments but also are time-consuming and laborious, which does not satisfy the demand for real-time analysis and greatly limits their application potential. Thus, it is highly imperative to develop a sensitive, rapid, and inexpensive method to detect clenbuterol.

Molecularly imprinted polymers (MIPs) are artificial, tailor-made receptors having specific molecular recognition properties for target analytes, which are formed by polymerizing functional monomers with template molecules in the presence of cross-linkers.[11–13] MIPs have been widely used for chromatographic separation, enzyme-mimicking catalysts, artificial antibodies, and sensors,[14–18] because of their considerable advantages of long stability, low cost, and ease of preparation in comparison with natural recognition agents. Molecular imprinting has already been used for extraction and detection of clenbuterol.[19–22]

In recent years, MIPs have been employed in surface plamson resonance (SPR) sensors, to serve as the sensing element.[23–26] The SPR sensor, an optical technique that measures changes in refractive index within the evanescent field near a metal surface, can transduce binding events between MIPs and its target molecules into optical signals.[27,28] Detections of clenbuterol using SPR sensor have been reported, and nanomolar detection levels have been achieved. Johansson et al.[29] applied SPR sensors in antibody-based inhibition assay for analysis of clenbuterol in cattle hair. The limit of detection (LOD) of this method was 3.6×10^{-8} mol L^{-1}. Traynor et al.[30] used a β-agonist monoclonal antibody in an immuno-biosensor assay based on SPR and obtained the detection limit of clenbuterol at 4×10^{-10} mol L^{-1}. Up to now, however, it has still not been reported in the literature an SPR sensor using MIPs for detection of clenbuterol.

The objective of our present work was to integrate molecularly imprinted film (MIF) with an SPR transducer to detect clenbuterol. The MIF was prepared by thermal polymerization on the gold surface of a sensor chip, providing a sensing element with targeted selectivity. A scanning electron microscope (SEM) was used to characterize the structure and thickness of MIF. The clenbuterol-binding properties of MIF was directly monitored by SPR sensor.

The MIF was prepared by thermal copolymerization of methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) in the presence of clenbuterol as template molecules on bare gold surface of SPR substrates as described in ref 18 with some modifications. The SPR spectrometer used in this study (Scheme 1) was homebuilt. For details of MIF preparation and SPR instruments, please see Supporting Information.[31] To evaluate binding properties of MIF, the experiments of template removal were first carried out by injecting 5 mL of acetonitrile/acetic solution in 9:1, 8:2, and 7:3 volume ratios. Each eluent was injected for 10 min, and then the SPR response was measured in the acetonitrile to assess the removal efficiency. In the adsorption experiments, acetonitrile solutions of clenbuterol with a series of concentrations from 10^{-15} to 10^{-3} mol L^{-1} were successively injected into the flow cell at the flow rate of 0.5 mL/min. Each injection lasted for 15 min at least to reach



Scheme 1. Schematic drawing of the MIF-based SPR sensor for detection of clenbuterol.

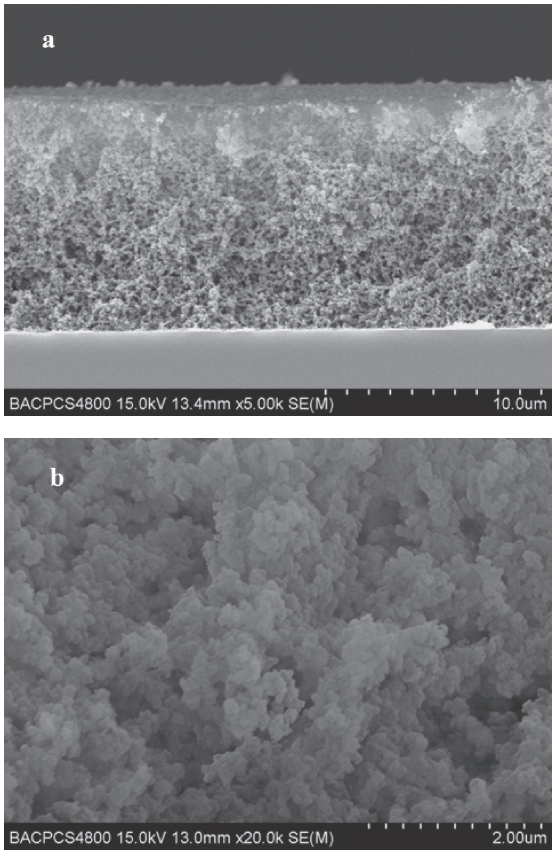


Figure 1. SEM images of the MIF-coated sensor chip: (a) cross-senctional view, (b) amplified view of the film surface.

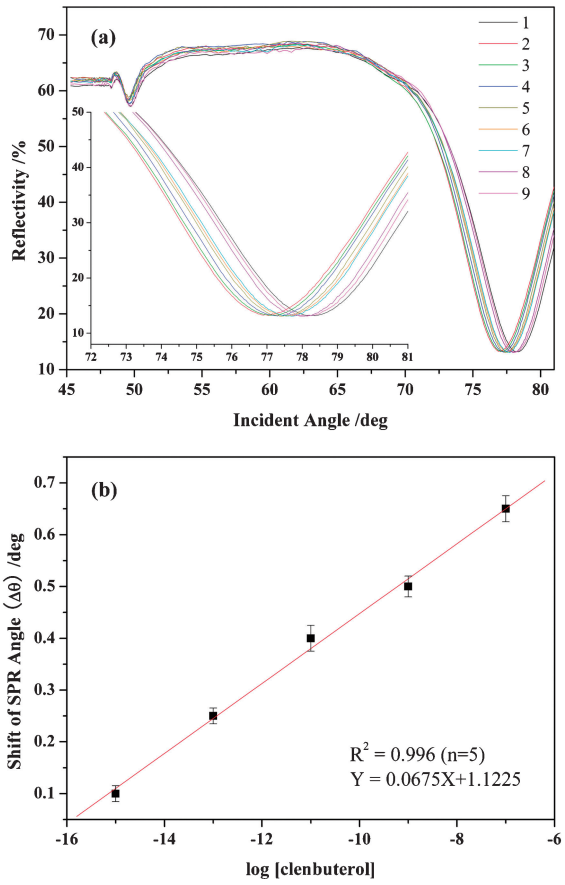


Figure 2. (a) SPR response for the rebinding of different concentration of clenbuterol with MIF, 1) before rinse, 2) after rinse, 3) 10^{-15}, 4) 10^{-13}, 5) 10^{-11}, 6) 10^{-9}, 7) 10^{-7}, 8) 10^{-5}, and 9) 10^{-3} mol L^{-1}. Inset: the enlarged view around resonance angle. (b) Calibration plot for the rebinding of clenbuterol, fitted with linear function. Error bars are the standard deviation for 3 trials.

equilibrium adsorption, and then the film was rinsed with acetonitrile for 6 min.

The clenbuterol-imprinted film was characterized by SEM. As shown in Figure 1a, the polymer layer appeared to be successfully immobilized on bare gold surface; the thickness of membrane was approximately 10 μm. Moreover, the micrograph of surface morphology (Figure 1b) revealed that the polymer film was crosslinked with a lot of micropores distributed in it. This porous structure was supposed to enable access of clenbuterol to the binding sites easily.

Subsequent removal of the templates from the imprinted film following the polymerization process leaves binding sites within the polymer possessing both shape and the correct orientation of functional groups, which are capable of selectively recognizing the imprint species. Degree of template removal is very crucial for the binding properties of MIF. In this experiment, the amino group and hydroxy group in clenbuterol could form hydrogen bonds with the functional monomer MAA.[19] Thus, the clenbuterol molecules were removed by repeated extraction with the mixtures of acetonitrile and acetic acid with an increasing portion of acid. The SPR angular reflectivity spectra before and after rinsing with 9:1 volume ratio of acetonitrile/acetic solution are shown by curve 1 and 2 in Figure 2a. The removal of template clenbuterol caused a decrease in refractive index near the sensor chip, shifting the resonance angle of 1.3° to the left from 78.2 to 76.9°. No shift was observed in the resonance angle associated with an increment in the proportion of acetic acid in eluent (data not shown), suggesting that an efficient removal of the template was achieved by the volume ratio of 9:1. We found that the degree of crosslinking of imprinted film was very important for the SPR sensing in this experiment. When the mole ratio of crosslinker to functional monomer used for polymerization was lower than 6:3 or higher than 10:3, the sensitivity and capacity of imprinted film to clenbuterol decreased dramatically. The observed decreases may be due to the less homogeneous and fragile film at low crosslinked structure and incomplete washing for the template and poor efficiency in clenbuterol rebinding at high crosslink structure. Thus, the mole ratio of crosslinker to functional monomer used in the experiment was 8:3.

The molecular recognition properties of the imprinted films were evaluated by SPR measurements of rebinding capacities to clenbuterol molecules. Figure 2a shows the SPR response for various concentration of clenbuterol in acetonitrile soluiton. The SPR angles shifted to higher value with increasing concentration of clenbuterol due to the rebinding of clenbuterol molecules to imprinted sites. As illustrated in Figure 2b, the calibration curve obtained by plotting the resonance angle changes ($\Delta\theta$) versus

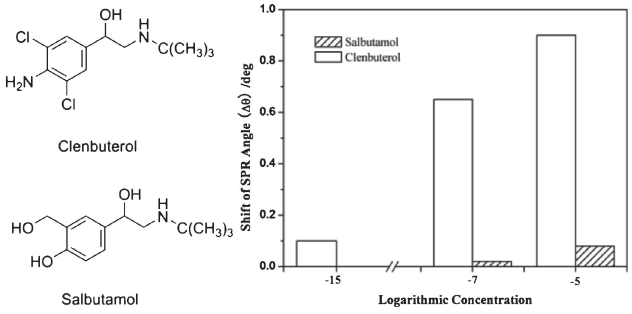


Figure 3. Interference effects of salbutamol, in comparison with clenbuterol.

logarithmic concentration of clenbuterol showed a high linearity ($R^2 = 99.6\%$) from 10^{-15} to 10^{-7} mol L^{-1}. Here, the $\Delta\theta$ is the difference in resonance angles before adsorption and after adding a certain concentration of clenbuterol solution. On the other hand, in the case of nonimprinted film, no perceptible change in SPR signal was observed at the concentration of 1×10^{-5} mol L^{-1}, which indicated that no specific and non-specific binding of clenbuterol occurred in the nonimprinted film had taken place. This result suggested that the sensor chip possessed good recognition and very low LOD toward clenbuterol.

The salbutamol (see the chemical structure in Figure 3) was employed as an analogue of clenbuterol to evaluate the selectivity of the MIF. The changes in the resonance angle of clenbuterol were compared with those of salbutamol after injection of their acetonitrile solutions. As shown in Figure 3, there was no obvious response in the resonance angle to salbutamol until the concentration is as high as 10^{-5} mol L^{-1}. This suggests that the MIF had specific binding affinity to clenbuterol.

In this study, we combined MIF which functions as a recognition element with an SPR sensor to detect clenbuterol. The LOD obtained was 1.0×10^{-15} mol L^{-1}, and the SPR sensor exhibited a wide range of linear response from 1.0×10^{-15} to 1.0×10^{-7} mol L^{-1}. Whereas, with regard to salbutamol, no response was observed below 1.0×10^{-5} mol L^{-1}. This MIF-based SPR sensor with high sensitivity and selectivity provides a promising method for determinations of clenbuterol and other β-agonists.

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