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Mechanism of Surface Plasmon Resonance Sensing by Indirect Competitive Inhibition Immunoassay Using Au Nanoparticle Labeled Antibody

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Abstract

We investigated the use of a surface plasmon resonance (SPR) biosensor using an antibody (Ab) labeled with Au-nanoparticle (Ab-AuNP conjugate). As clenbuterol is a small molecule, an indirect competitive inhibition immunoassay was used. The SPR immunoassay using Ab-AuNP conjugate had an extremely low limit of the detection (LOD) with a magnitude of 50 ppq (50 fg mL^{-1}), which was 40 times lower than that of unlabeled Ab. To identify the key factor in determining the LOD of the indirect competitive inhibition immunoassay, kinetic equations described using the affinity constants of the surface immunoreaction (K_1) and of the premixed solution (αK_2) were derived. We found that the localized surface plasmon resonance (LSPR) signal amplification did not affect on the kinetic factors, because all the amplification magnitude terms canceled out in the equations. Thus, the K_1 and αK_2 values were determined to $3.0 \times 10^{11} \text{ M}^{-1}$ and $2.9 \times 10^{12} \text{ M}^{-1}$, respectively, which were three and four orders of magnitude higher, respectively, than those of unlabeled Ab. The simulation plot of LOD with respect to K_1 and αK_2 showed that a K_1 one order of magnitude lower than αK_2 produced a ppq-level LOD. Because the affinity constants are determined by the molar concentrations of reactant and product, the molar mass of the Ab or Ab-AuNP conjugate in the sample solution containing 1 ppm ($1 \text{ } \mu\text{g mL}^{-1}$) highly affects the constants. Consequently, molar mass adjustment can be used to adjust the LOD in an indirect competitive inhibition immunoassay as needed for a practical application.

Keywords: surface plasmon resonance; indirect competitive inhibition immunoassay; Au-nanoparticle; limit of detection

1. Introduction

Surface plasmon resonance (SPR) biosensors have been investigated as powerful tools for real-time and label-free monitoring since they were first introduced by Nylander and Leidberg in the early 1990s [1-2]. There are many review articles about SPR biosensors for environmental monitoring, fundamental biological studies, food safety, and clinical diagnosis [3-10]. However, only a few of these biosensors are commercially available [11]. The main challenge in the practical use of commercial SPR biosensors is posed by impurities in real samples, which interfere with the SPR signal [12]. Sample dilution is one approach that can be used to avoid impurity interference. However, it is difficult for SPR biosensors to directly detect analytes at extremely low (sub-nanomolar) concentrations because the sensitivity is determined not only by the instrument but also by the biochemical reaction. In other words, the kinetic factors of the biochemical reaction on the sensor surface also determine the sensitivity [13]. Thus, a practical biosensor requires a much lower limit of detection (LOD) than that achieved under ideal conditions. Therefore, we investigated sensitivity enhancement of a biosensor in the present study.

Various immunoassay formats have been applied to SPR biosensors for the detection of chemical and biological analytes. The most frequently used formats are direct immunoassay and sandwich immunoassay [3]. A direct immunoassays use an antibody (Ab)-immobilized sensor surface; an analyte is injected and reacts with Ab on the sensor surface. This format is preferred for large molecule detection. The signal and LOD can be improved using a secondary Ab because SPR senses the dielectric constant of the secondary Ab. The “Ab–analyte–Ab” structure is termed as a sandwich immunoassay. This immunoassay is widely used for large molecules having multiple binding sites, such as proteins, nucleic acids, bacteria, and viruses [6,14]. In

contrast, the detection of small analytes [molecular weight (MW) < 1000 g mol⁻¹] remains challenging because a small molecule cannot generate a sufficient response to the dielectric constant in SPR sensing. Indirect competitive inhibition immunoassays are useful for the detection of small molecules [15-21]. The target molecule is immobilized on Au surface, which are used together as the sensor chip. As the premixed solution of the Ab and the analyte is injected onto the sensor surface, SPR monitors the binding event of a high molar mass Ab instead of the analyte. Therefore, a high SPR signal change is expected.

Recently, to improve the LOD and SPR signal, localized surface plasmon resonance (LSPR) detection using Au-nanoparticles (AuNP) has drawn much attention by researchers [22-25]. Typically, two strategies are considered for SPR signal amplification: (1) sensor surface modification with AuNP and (2) labeling of a biorecognition part (such as an Ab) with AuNP. Both strategies use the coupling of LSPR and SPR with AuNP for signal and sensitivity improvement [24]. Fig. 1 shows the calibration curves in the presence or absence of AuNP on the sensor surface. In a direct immunoassay (Fig. 1A), Liu et al. demonstrated the detection of atrazine. They noticed that AuNP enhanced the SPR signal and that the LOD determined at the minimum value of the detectable range was reduced. On the other hand, AuNP-modified sensor surface has been used for the detection of 2, 4, 6-trinitrotoluene in an indirect competitive inhibition immunoassay (Fig. 1B) [22]. The calibration curve of the indirect competitive inhibition immunoassay was the sigmoid type. Although the SPR signal was enhanced by the LSPR effect, LOD was unchanged. Improvement in LOD of an indirect competitive inhibition immunoassay means that the calibration curve needs to be shifted toward a lower analyte concentration. This shift is determined by the kinetic factors of immunoreactions. AuNP labeling of Ab is mainly

used in sandwich immunoassays. Several researchers have reported the achievement of high sensitivities in the nanomolar to femtomolar range [23, 26-27]. To date, there have been no reports on an indirect competitive inhibition immunoassay using Ab modified with AuNP as the signal amplification strategy.

In this study, we investigated the use of Ab modified with AuNP in an indirect competitive inhibition immunoassay for detecting clenbuterol (MW, 277 g mol⁻¹). The target analyte, clenbuterol is a member of the β -agonist family and is a problematic material for food safety. Although, commercial test kit can detect clenbuterol at a detection level of 25 ppt (25 pg mL⁻¹) in meat samples by enzyme-linked immunoassay (ELISA) [28], however, this technique is time (>1 h) and reagent consuming. Therefore, it is of vital importance to develop a detection process that is rapid and cost effective. Recently, we reported an LOD of 2 ppt for detecting clenbuterol using an indirect competitive inhibition immunoassay [29]. However, this sensitivity is insufficient for the real sample detection in diluted solution. Even worse, the maximum change (5 mdeg) is too small for the miniaturized SPR instrument. Therefore, we investigated a signal amplification strategy using the Ab modified with AuNP in this study.

2. Materials and methods

2.1. Materials and instrumentation

All commercially available chemicals were analytical grade and used as received without further purification. Clenbuterol hydrochloride and the corresponding Ab [mouse immunoglobulin (IgG) clenbuterol, clone 1F8B10B7] were supplied by LKT Laboratories, Inc. (USA) and Novus Biologicals (USA), respectively. Phosphate-buffered saline (PBS, pH 7.4), 3,3'-dithiobis sulfosuccinimidyl propionate (DTSSP), and dithiobis succinimidyl propionate (DSP) were supplied by Thermos

Scientific (USA). Colloidal Au (40 nm) was purchased from British BioCell International Solutions (UK). Borate-buffered saline (pH 8.5) and 10% bovine serum albumin (BSA) solution were purchased from KPL (USA) and Sigma–Aldrich (USA), respectively. Ethanolamine, methanol, and ethanol were supplied by Kanto Chemical Co., Inc. (Japan), Dojindo (Japan), and WAKO (Japan), respectively. Sodium chloride and sodium hydroxide were purchased from Junsei Chemical Co., Ltd. (Japan). Refractive index-matching fluid (refractive index = 1.518) was obtained from Cargille Labs (USA). Deionized water (18.2 MΩ cm) was produced from a Millipore Milli-Q purification system.

2.2. Preparation of Au substrates

Glass chips (BK7, refractive index = 1.515, 20 mm × 13 mm × 0.7 mm from Matsunami Glass Ind., Ltd.) were used as the SPR sensor chips. These chips were cleaned with soapy water (10% Contrad 70, Decon Laboratories, Inc.) and acetone in an ultrasound bath for 30 min. After this process, the glass chips were rinsed using a copious amount of Milli-Q water. Then, the chips were dried using pure nitrogen gas (purity > 99.995%), following which light treatment (deep UV, $\lambda = 172$ nm) was performed to produce a hydrophilic surface. The glass chips were then placed in a sputtering system (JEOL, Japan). 50 nm Au film was deposited using the sputtering method. Sputtering was performed under 2.0–2.5 Pa. Finally, the prepared gold chip was loaded into the SPR instrument immediately after sputtering.

2.3. Preparation of AuNP-labeled Abs

In this study, Abs covalently modified with AuNP (Ab-AuNP conjugate) was used for detection in an indirect competitive inhibition immunoassay. The 40-nm size

of AuNPs was chosen because it provides the highest coupling effect between the LSPR of AuNP and SPR of the Au substrate [30].

The Ab-AuNP conjugate was prepared according to the literature [31]. pH of the colloidal-Au solution (1 mL) was adjusted to 8.5 with 40 μ L of 50 mM borate buffer (pH 8.5). Then, 10 μ L of 1 mM DTSSP in aqueous solution was added to the AuNP suspension. After 30 min of mixing at room temperature, the sulfo-*N*-hydroxy succinimidyl (NHS)-terminated AuNP suspension was centrifuged for 5 min at 7000 $\times g$ to separate excess and unreacted DTSSP. AuNP was then resuspended in 2 mM borate buffer, and primary Ab (20 μ g) was added. In this step, sulfo-NHS-terminated AuNP covalently binds to the side chain amino group of Ab. After standing for 90 min, the mixture was then centrifuged at 7000 $\times g$ for 5 min. The supernatant was discarded. Next, the Ab-AuNP conjugate was resuspended in 2 mM borate buffer containing 1% BSA. The processes of resuspension and centrifugation were repeated once again to effectively remove excess reagent. The suspension was incubated for 30 min to allow BSA to block any unreacted sulfo-NHS ester and nonspecific binding sites. A small volume of concentrated NaCl (10%) was added to the suspension to reach a final concentration of 150 mM. Finally, a PBS + 10% ethanol solution was added to achieve the desired concentration. The prepared Ab-AuNP conjugate was either used immediately or stored overnight at 4°C.

2.4. Characterization of the Ab-AuNP conjugate

The Ab-AuNP conjugate solution was red because of light absorption in a specific wavelength range (Fig. S1 in the Supporting Materials). Measurement of the light absorbed by the AuNP suspension using a UV-Vis spectrometer (U-3310 spectrophotometer; Hitachi, Japan) showed a maximum absorption peak at 525.0 nm

1 before conjugation, whereas the absorption peak was shifted to 531 nm for the Ab-
2 AuNP conjugate solution. This shift was caused by a change in the local refractive
3 index due to binding of Ab onto the sulfo-NHS end.
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10 2.5. SPR measurements

11 SPR experiments were performed using an SPR analyzer (SPR-670; Nippon
12 Laser Electronics, Japan). The instrument was equipped with a computer-controlled
13 flow injection system for immunoanalysis based on a Kretschmann-attenuated total
14 reflectance (ATR) configuration. A prepared sensor chip (glass chip coated with an Au
15 layer of 50 nm thickness) was mounted on the semi-cylindrical prism (refractive
16 index = 1.515) of the SPR analyzer using a refractive index-matching fluid to
17 decrease the deformation of SPR images. A red light-emitting source (Ni-Cd laser
18 beam, $\lambda = 670$ nm) reflected light onto the sensor chip at ATR angles, and the
19 intensity of reflected light was recorded using a charge-coupled device camera. The
20 reflectance angle at which the light intensity is the minimum, called the SPR angle,
21 was recorded with respect to time on a personal computer. All the experiments were
22 performed in an air-conditioned room ($25 \pm 1^\circ\text{C}$).
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41 2.6. Fabrication of immunosurface

42 Fig. 2 illustrates the fabrication process of the immunosurface according to the
43 self-assembly method. First, methanol was flowed over the gold surface as a carrier
44 solution. Once a stable baseline was achieved, a 5 mM DSP methanolic solution was
45 injected onto the Au chip for 40 min at a flow rate of $5 \mu\text{L min}^{-1}$. The Au surface was
46 covered with a succinimidyl-terminated propenethiol monolayer. Clenbuterol (1000
47 ppm) in PBS solution was then injected onto the surface for 40 min. The NHS group
48 was replaced with clenbuterol through an amide coupling reaction. Finally, to block
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unreacted NHS groups, ethanolamine (1000 ppm) in PBS solution was flowed over the surface as a blocking agent. The surface concentrations of succinimidyl propanethiol, immobilized clenbuterol, and ethanolamine were calculated from the observed angle shift as $3.3 \pm 1.2 \times 10^{-10} \text{ mol cm}^{-2}$, $1.1 \pm 0.3 \times 10^{-10} \text{ mol cm}^{-2}$, and $1.8 \pm 0.4 \times 10^{-10} \text{ mol cm}^{-2}$, respectively. The reproducibility was confirmed by performing the same experiment five times.

3. Results and Discussion

3.1. Immunoassay using the Ab-AuNP conjugate

Clenbuterol detection was performed using an indirect competitive inhibition immunoassay and Ab-AuNP conjugate. Fig. 3 shows the SPR sensogram recorded during immunosensing detection of the Ab-AuNP conjugate (1 ppm) for 0 to 1 ppb (1 ng mL^{-1}) of clenbuterol. Because of the refractivity difference between the running buffer (PBS) and sample solution, the SPR signal abruptly decreased after sample solution injection. For the calibration curve (Fig. 4), the angle shifts were calculated from the difference in the SPR signal before and after sample injection (from $t = 150 \text{ s}$ to $t = 450 \text{ s}$). As shown in Fig. 3 (inset), the signal slightly decreased until regeneration solution injection because of the desorption of nonspecifically adsorbed Ab-AuNP conjugate from the sensor surface. The amount of nonspecifically adsorbed Ab-AuNP conjugate was estimated to be approximately $12.6 \pm 4.0\%$. In support of this view, there is a recent report suggesting that the orientation of Ab molecules on the nanoparticle surface is not uniform [32]. For example, one set of Abs that is outwardly oriented on the AuNP surface has a high affinity for capturing analytes onto the sensor surface. Another set of Abs may be immobilized onto the AuNP surface with less affinity for antigen binding. Because the immobilized analyte is

much smaller than Ab-AuNP, some conjugates may be deposited on the sensor surface without strongly binding to the sensor surface because of the orientation of the Ab molecule on the nanoparticle surface. Therefore, the unbound or loosely bound conjugates are desorbed from the sensor surface when the running solution is switched from the sample solution to PBS.

It was also found that a regeneration solution of 0.1 M NaOH removed all Ab-AuNP conjugates from the sensor surface. The sensor surface could be used again, as illustrated in Fig. 3 (inset). The surface is robust because the sensor surface architecture consists of covalent bonds (S-Au, S-S, and NHCO).

A typical sigmoidal-type calibration curve of the indirect competitive inhibition immunoassay was obtained and is shown in Fig. 4. Because the amount of free Ab-AuNP conjugate decreased with increasing clenbuterol concentration, the angle shifts also decreased with increasing clenbuterol concentration. The highest angle shift (14.8 mdeg) was observed in the absence of clenbuterol because the free Ab-AuNP concentration was at the maximum. The angle shift was approximately 3-fold higher than that of unlabeled Ab (5 mdeg). This signal enhancement was caused by the LSPR effect and dielectric constant difference between unlabeled Ab and the Ab-AuNP conjugate.

The LOD has been typically defined at 85% of the maximum angle shift [15, 22, 28]. LOD of the immunoassay for the detection of clenbuterol was determined to be 50 ppq (50 fg mL⁻¹) using the Ab-AuNP conjugate. In contrast, LOD was only 2 ppt (2 pg mL⁻¹) for the unlabeled Ab, which was approximately 40 times higher than that of the Ab-AuNP conjugate. To the best of our knowledge, such extremely high sensitivity has not been reported for the detection of clenbuterol [ELISA: 100–25 pg mL⁻¹ [27, 33]; amperometry: 1.3 pg mL⁻¹ [34]; fluorescence spectroscopy: 0.12 µg

L^{-1} [35]; SPR: $2.0 \mu g L^{-1}$ [36]; Spectral Imaging SPR: $6.7\text{--}4.5 \mu g mL^{-1}$ [37].

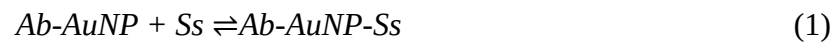
Sensitivity enhancement can be explained by kinetic analysis of the indirect competitive inhibition immunoassay. The detailed mechanism will be discussed in the next section.

3.2. Kinetic simulation

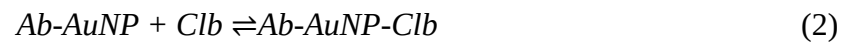
It is known that AuNP causes LSPR signal amplification. Thus, to date, kinetic simulation has not been performed because an accurate reactant amount cannot be estimated from the signal amplification. Therefore, the kinetic model of an indirect competitive inhibition immunoassay is reconfirmed from the basic Langmuir adsorption isotherm considered with LSPR signal amplification.

The angle shift is a result of biomolecular interactions during the capture of the Ab-AuNP conjugate by the analyte immobilized on the sensor surface. In the indirect competitive inhibition immunoassay, Ab-AuNP conjugate solution is premixed with clenbuterol solution; the Ab-AuNP conjugate reacts with clenbuterol immobilized on the sensor surface (Ss) and clenbuterol in the premixed solution (Clb).

Immunoreaction on the sensor surface can be denoted as follows:



Immunoreaction in the premixed solution can be expressed as:



The affinity constants for the surface immunoreaction and immunoreaction in the premixed solution were defined as K_1 and K_2 , respectively.

$$K_1 = \frac{[Ab - AuNP - Ss]}{[Ab - AuNP][Ss]} \quad (3)$$

$$K_2 = \frac{[Ab - AuNP - Clb]}{[Ab - AuNP][Clb]} \quad (4)$$

The surface immunoreaction of the Ab-AuNP conjugate with the sensor surface is modeled by the Langmuir adsorption isotherm. The Langmuir adsorption isotherm for SPR immunosensing is written as follows:

$$\frac{1}{\Delta\theta_{Ab-AuNP}} = \frac{1}{\Delta\theta_{Ab-AuNP,max}} + \frac{1}{K_1[Ab-AuNP]\Delta\theta_{Ab-AuNP,max}} \quad (5)$$

Here, $\Delta\theta_{Ab-AuNP}$ is the total angle shift for the standard concentration (10 ppb–1 ppm) of the Ab-AuNP conjugate and $\Delta\theta_{Ab-AuNP,max}$ is the SPR angle shift when the maximum amount of the Ab-AuNP conjugate is bonded to the reaction site on the sensor surface. It is assumed that the SPR angle is proportional to the amount of Ab-AuNP captured onto the adsorption site (immobilized clenbuterol), and it is thought that the reacted amount could not be estimated by LSPR signal amplification. Hence, the signal amplification magnitude is defined as “A”. It is assumed that A is always the same (constant) under our experimental conditions. It is reasonable to wonder whether this assumption is true. $\Delta\theta_{Ab-AuNP,max}$ is definitely overestimated because of LSPR signal amplification. Thus, the true maximum concentration must be lower than $1549 \mu\text{m}^2/\text{Ab-AuNP}$ estimated from $\Delta\theta_{Ab-AuNP,max}$ (108 mdeg). This value is too small to influence the particle resonance of the LSPR effect. Only the resonance between AuNP and the Au substrate needs to be considered. Therefore, it is reasonable to assume that “A” is always constant. Hence, Eq. (5) can be rewritten as follows:

$$\frac{1}{A\Delta\theta_{Ab-AuNP}} = \frac{1}{A\Delta\theta_{Ab-AuNP,max}} + \frac{1}{K_1[Ab-AuNP]A\Delta\theta_{Ab-AuNP,max}} \quad (6)$$

All the “A” terms can be canceled out in this equation. Therefore, it can be concluded that the LSPR signal amplification did not affect the determination of K_1 . Hence, K_1 was estimated to be $3.0 \times 10^{11} \text{ M}^{-1}$ (Fig. S2A in the Supporting Materials).

For the estimation of K_2 with “A,” the following kinetic equation can be used

(Suherman et al., 2015):

$$\frac{1}{A\Delta\theta} = \frac{1}{A\Delta\theta_{1ppm,Ab-AuNP}} + \frac{\alpha K_2[Clb]_0}{K_1[Ab-AuNP]_{total}A\Delta\theta_{Ab-AuNP,max}} \quad (7)$$

Because “A” can also be canceled in this equation, it is concluded that the LSPR effect does not affect on αK_2 as well as K_1 . αK_2 was calculated to be $2.9 \times 10^{12} \text{ M}^{-1}$ from the slope in Fig. S2B (in the supporting Materials). K_1 and αK_2 of unlabeled Ab were estimated to be $4.8 \times 10^8 \text{ M}^{-1}$ and $1.0 \times 10^8 \text{ M}^{-1}$, respectively (data not shown). Notably, K_1 and αK_2 of the Ab-AuNP conjugate were three and four orders of magnitude higher than those of unlabeled Ab, respectively. These differences in affinity constants can be easily explained by the molar concentration calculations of Eq. (3) and Eq. (4). The molecular mass of unlabeled Ab (MW 46.5 kg mol⁻¹) is four orders of magnitude lower than that of the Ab-AuNP conjugate (MW 390,000 kg mol⁻¹). In the premixed solution containing 1 ppm Ab or Ab-AuNP conjugate, the molar concentration of Ab-AuNP was four orders of magnitude lower than that of unlabeled Ab. Because [Ab-AuNP] was in the denominators of Eq. (3) and Eq. (4), the affinity constants became high. Size comparison revealed that [Ab-AuNP-Ss] is one order smaller than [Ab-Ss]. Thus, the K_1 and αK_2 values of the Ab-AuNP conjugate were three and four orders of magnitude higher than those of unlabeled Ab, respectively. Therefore, the molecular mass of the reactant has a greater effect than LSPR on the affinity constants.

Aiming to identify the determining factor of LOD, simulations were constructed. To compare the calibration curves, the SPR signals ($\Delta\theta$) were normalized to the percentage of the maximum angle shift ($\Delta\theta_{1ppm,Ab-AuNP}$) obtained in the absence of an analyte. The following equation can be derived from Eq. (7):

$$\frac{\Delta\theta}{\Delta\theta_{1ppm,Ab-AuNP}} = \left(1 + \frac{\alpha K_2[Clb]_0 \Delta\theta_{1ppm,Ab-AuNP}}{K_1[Ab-AuNP]_{total} \Delta\theta_{Ab-AuNP}} \right)^{-1} \quad (8)$$

The derivation procedure (S4) is mentioned in the Supporting Materials. Using Eq. (8),

the simulations of $\Delta\theta/\Delta\theta_{1ppm,Ab-AuNP}$ were plotted as functions of K_1 and αK_2 , as illustrated in Fig. 5. The experimental results show good agreement with the theoretical curve ($3.0 \times 10^{11} \text{ M}^{-1}$ and $2.9 \times 10^{12} \text{ M}^{-1}$ for K_1 and αK_2 , respectively).

We found that LOD shifted toward higher concentrations at higher K_1 values. In contrast, LOD shifted toward lower concentrations at higher αK_2 values. Thus, we concluded that the balance of K_1 and αK_2 determines LOD of the indirect competitive inhibition immunoassay. To support this conclusion, simulation of LOD was also performed. LOD was estimated according to the following equation:

$$\text{LOD} = \frac{15K_1[Ab - AuNP]_{total}\Delta\theta_{Ab-AuNP,max}}{85\alpha K_2\Delta\theta_{1ppm,Ab-AuNP}} \quad (9)$$

A detailed derivation is presented in the Supporting Materials. LOD is plotted according to Eq. (9) with respect to K_1 and αK_2 in Fig. 6. The plot clearly shows that higher values of K_1 and lower values of αK_2 lead to higher values of LOD. Because this is a rising diagonal slope from the bottom left to top right, the same LOD can be obtained from the same order of K_1 and αK_2 . To achieve LOD at the ppq (fg mL^{-1}) level, αK_2 must be one order of magnitude higher than K_1 . Thus, to prove the concept of LOD determination, further experiments such as those on the size dependence of AuNPs for Ab labeling may be needed.

In summary, Ab labeled with AuNP in an indirect competitive inhibition immunoassay was examined with the aim to enhance the SPR signal change and the sensitivity. LOD of the immunoassay for the detection of clenbuterol was determined to be 50 ppq (50 fg mL^{-1}) using the primary Ab-AuNP conjugate. In contrast, LOD was only 2 ppt (2 pg mL^{-1}) for the unlabeled primary Ab, which was approximately 40 times higher than that of the Ab-AuNP conjugate. LOD is influenced by the balance between K_1 and αK_2 . In particular, LOD achieved for αK_2 was one order of magnitude higher than that achieved for K_1 . We propose that LOD can be adjusted to

a required level because the affinity constants (K_1 and αK_2) are determined by the molar concentrations of the reactant and sensor surface ($\Delta\theta$ and $\Delta\theta_0$).

4. Conclusion

This study aimed to develop a method for highly sensitive detection of clenbuterol. To achieve this goal, AuNP was used as label for antibody in an indirect competitive inhibition immunoassay. A sensitivity of 50 ppq (50 fg mL^{-1}) was achieved, which was the highest among the past reports using SPR, ELISA, amperometry and fluorescence spectroscopy. The sensitivity mechanism was elucidated by kinetic analysis, which showed that LSPR signal amplification did not affect the affinity constants (K_1 and αK_2) because the LSPR amplification magnitude terms cancel out in the Langmuir adsorption equation. Therefore, the Langmuir adsorption isotherm for SPR sensing could be used for the calculation of K_1 and αK_2 , which were estimated to be $3.0 \times 10^{11} \text{ M}^{-1}$ and $2.9 \times 10^{12} \text{ M}^{-1}$, respectively. The affinity constants were three and four orders of magnitude, respectively, higher than those of unlabeled Ab. The enhancement of the affinity constants was caused by the molar concentration difference between the Ab-AuNP conjugate and unlabeled Ab in 1 ppm ($1 \text{ } \mu\text{g mL}^{-1}$) of premixed solution. Thus, we concluded that sensitivity enhancement is caused by molar concentration difference. To understand the mechanism underlying changes in LOD, simulation was performed. We found that the requirement of LOD at the ppq level is that αK_2 must be one order of magnitude higher than K_1 . The results showed that LOD can be adjusted to a required level because the affinity constants can be adjusted by changing the molar masses of the reactant (Ab).

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Figure legends

Scheme 1. Comparison of calibration curves for the AuNP-labeled and unlabeled SPR biosensor

(A) Direct immunoassay (Liu et al., 2015) and (B) indirect competitive inhibition immunoassay (Kawaguchi et al., 2008).

Fig. 1. SPR sensogram recorded during the fabrication process of the sensor surface

(a) Self-assembly process of the succinimidyl-terminated propenethiol (DSP) monolayer, (b) immobilization of clenbuterol (Clb) by amide coupling reaction, and (c) blocking of the unreacted *N*-hydroxysuccinimidyl group with ethanolamine. Flow rate was 5 $\mu\text{L min}^{-1}$.

Fig. 2. SPR sensogram of the immunoreactions with clenbuterol and Ab-AuNP conjugate

Premixed solutions were comprised 1 ppm Ab-AuNP conjugate and the clenbuterol standard solutions [(A) 0 ppt clenbuterol and (B) 0.01 ppt, (C) 0.1 ppt and (D) 1 ppt clenbuterol]. Inset figure shows the full time-course measurement. Regeneration solution was 0.1 M NaOH. The flow rate of the carrier solution was 100 $\mu\text{L min}^{-1}$.

Fig. 3. Calibration curves for the detection of clenbuterol using unlabeled Ab and Ab-AuNP conjugate

Fig. 4. Simulated calibration curves: Ab-AuNP conjugate in the indirect competitive inhibition immunoassay

Filled triangles are experimental data for Ab-AuNP. The simulation curves are calculated using Eq. (7), assuming (A) $\alpha K_2 = 2.9 \times 10^{12} \text{ M}^{-1}$ and (B) $K_I = 3.0 \times 10^{11} \text{ M}^{-1}$.

Fig. 5. Simulation of LOD based on Eq. (8) with respect to K_I and αK_2

LOD is calculated from Eq. (9),
$$\text{LOD} = \frac{15K_1[Ab - AuNP]_{total}\Delta\theta_{Ab-AuNP,max}}{85\alpha K_2\Delta\theta_{1ppm,Ab-AuNP}}.$$

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Figure 1

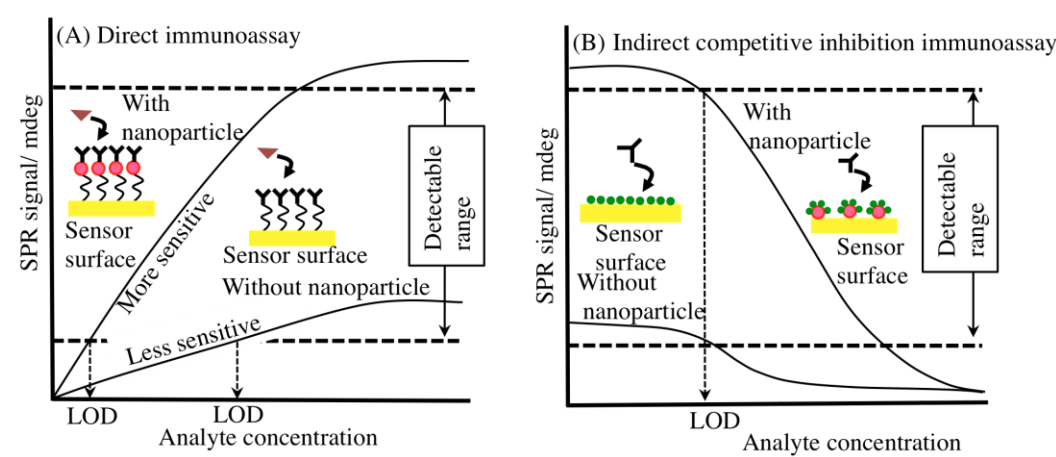


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Figure 2

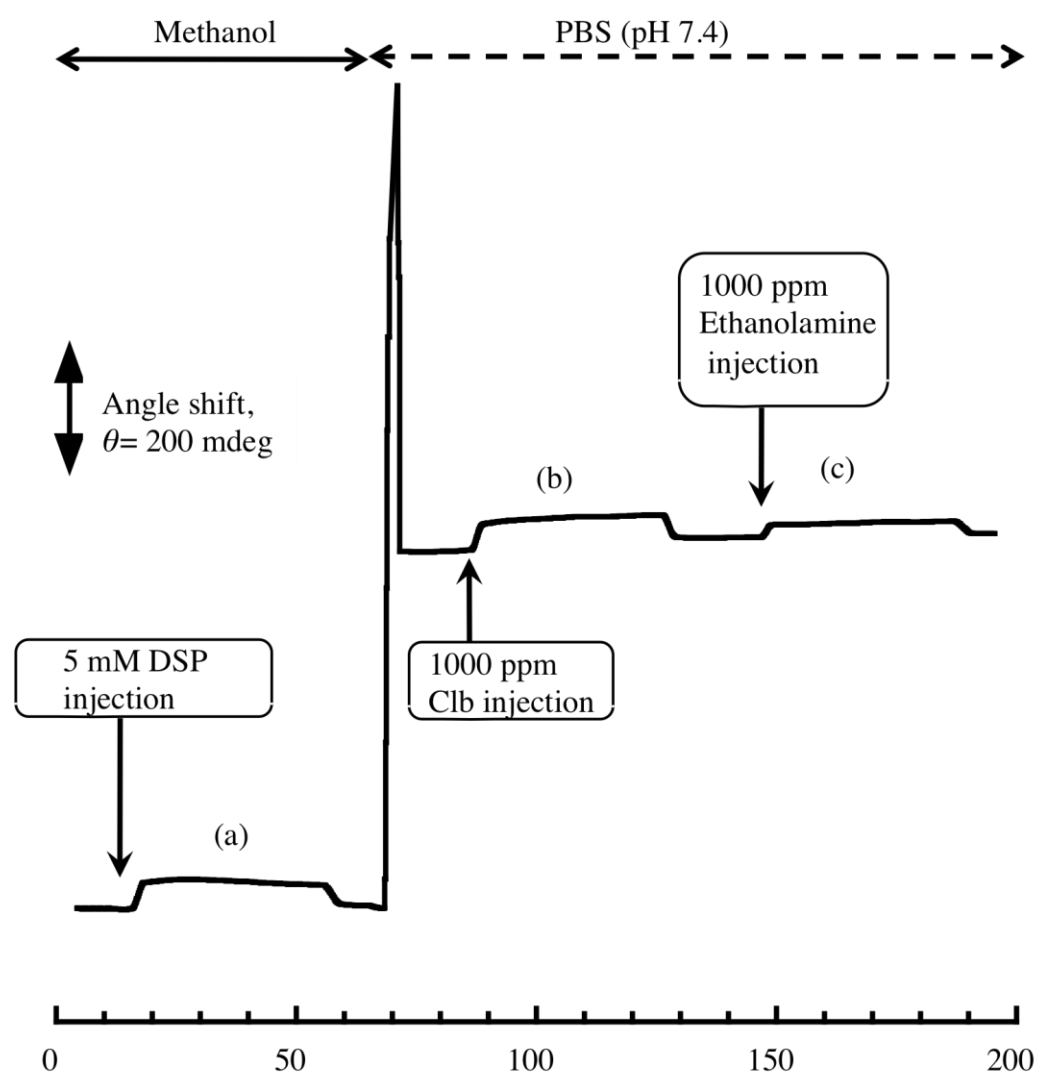


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Figure 3

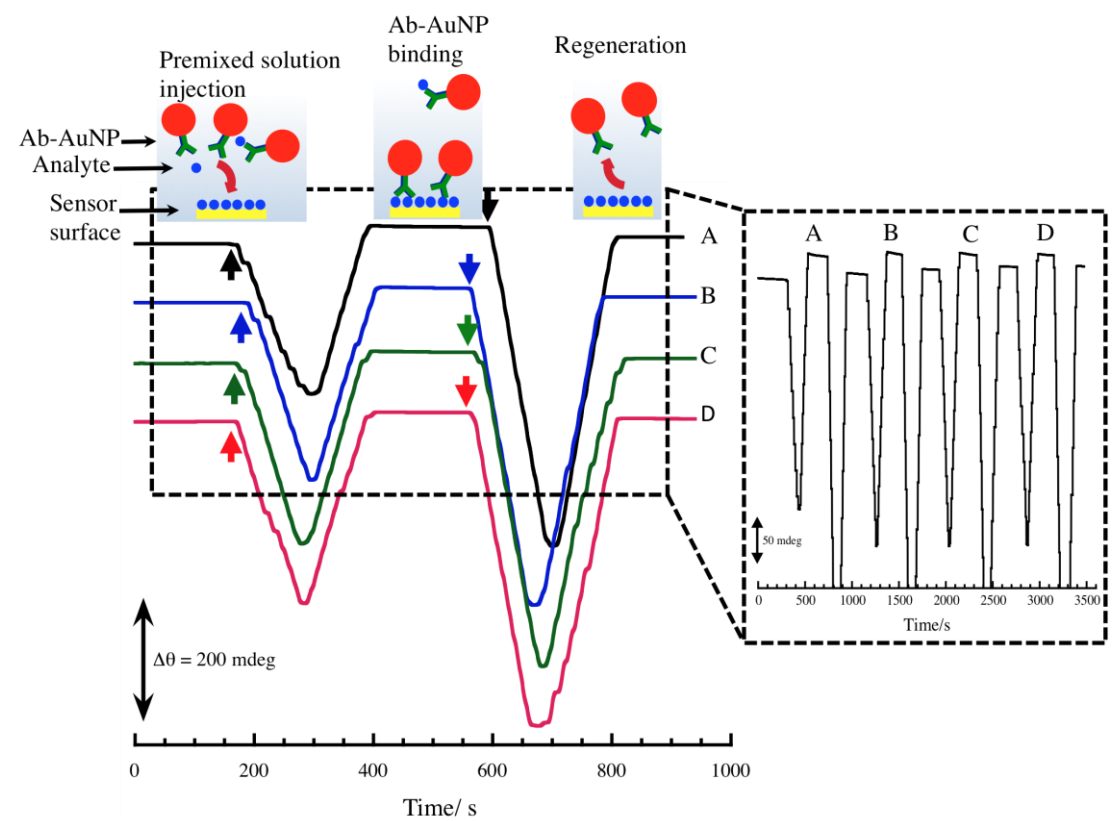


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Figure 4

Figure 4

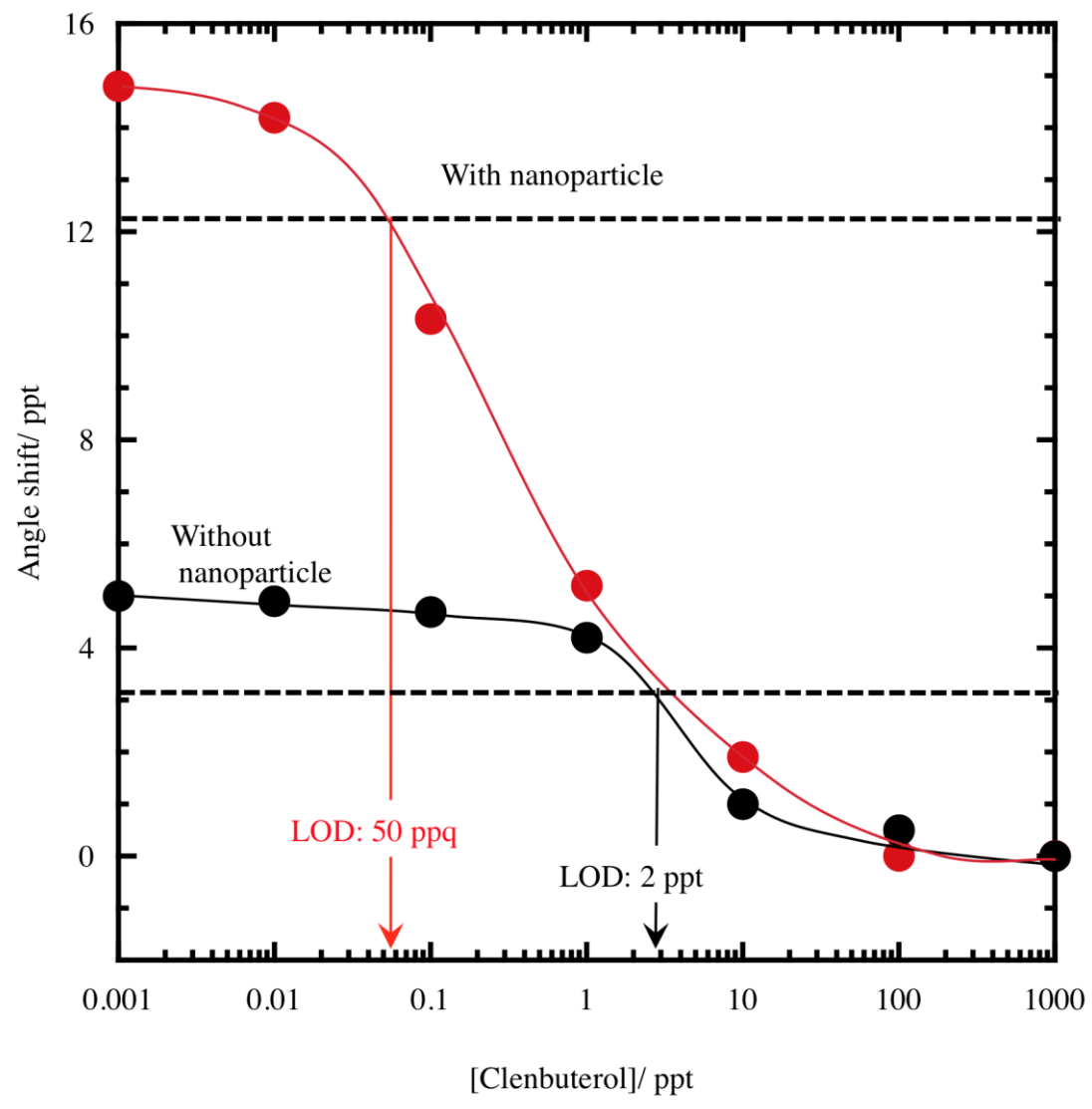


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Figure 5

Figure 5

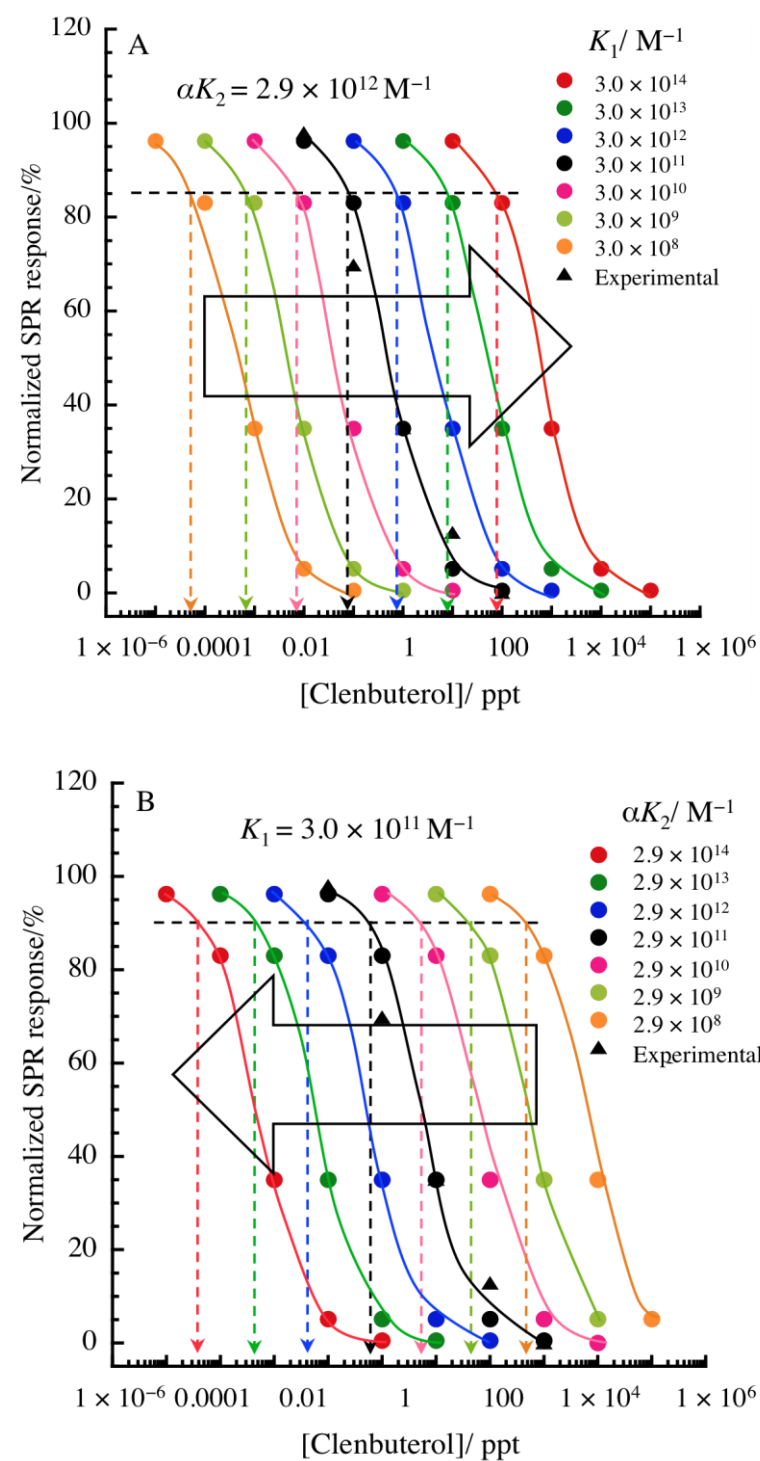


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Figure 6

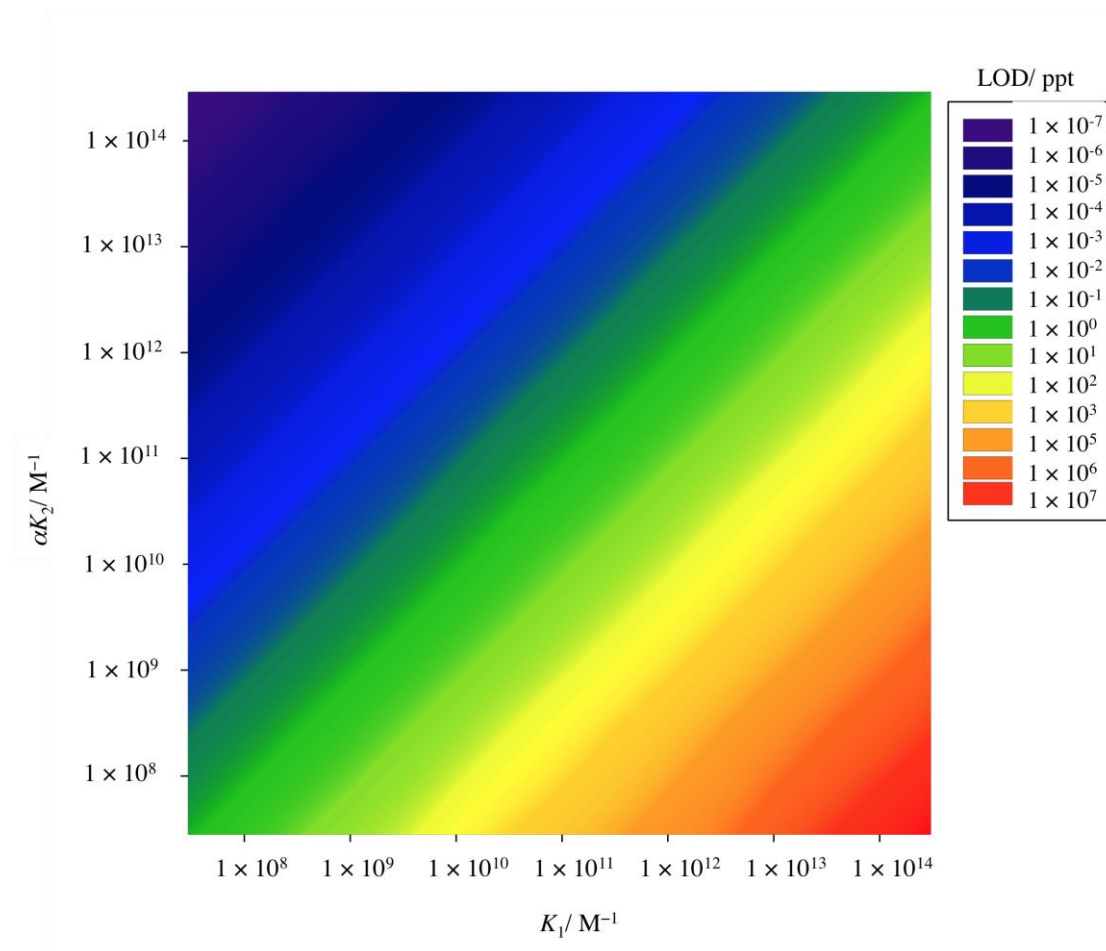


Fig. 6. Simulation of LOD based on Eq. (8) with respect to K_1 and αK_2

LOD is calculated from Eq. (9),
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Mechanism of Surface Plasmon Resonance Sensing by Indirect Competitive Inhibition Immunoassay Using Au nanoparticle Labeled Antibody

Graphical Abstract

