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ROYAL SOCIETY OF CHEMISTRY

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DOI: 10.1039/C9AN01183H

ARTICLE

Sequential sandwich immunoassay for simultaneous detection in trace samples using single-channel surface plasmon resonance

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

To analyze multiple analytes in trace samples, the low-dosage usage and high efficiency become crucial in many common cases. Here, we developed a facile method using a single-channel surface plasmon resonance (SPR) based biosensor for a simultaneous detection of gentamicin (GEN) and melamine (MEL) in milk and serum with only one sample injection. Based on a sandwich immunoassay, non-interfering antibodies against GEN from mouse (AbGEN) and against MEL from rabbit (AbMEL) were chosen to capture analytes. Second antibodies against mouse (AbM) and rabbit (AbR) were used to bind with AbGEN and AbMEL to determine the concentrations of GEN and MEL on a single channel of SPR sensor. All the detection process can be done in 10 minutes with 50 µL of sample injection. According to the response shifts of AbM and AbR, two standard curves for GEN and MEL were obtained successively with the limit of detection (LOD) values at 4.4 ng/mL and 1.3 ng/mL, respectively. Moreover, the feasibility was determined by spiking milk and serum samples with GEN and MEL, with recoveries of 81.6% - 118.0%. Importantly, the analytes can be substituted by others for much more applications, this method is also expected to multiply the detection efficiency of multi-channel SPR biosensors with low-dosage samples in the future.

Introduction

Surface plasmon resonance (SPR) based biosensor is a powerful analytical technique for detection of chemical and biological analytes[1]. Based on the local change in refractive index, SPR biosensors are used to monitor molecular interactions and determine the corresponding mass change on the sensor surface[2, 3]. Usually, biorecognition elements are immobilized on sensor surfaces to capture analytes, providing a real-time monitor of interaction between ligands and analytes[4]. With the advantages of non-labelling and high sensitivity, SPR biosensors have been widely applied in food safety[5, 6], disease diagnosis[7, 8], drug screening[9, 10] and environmental monitoring[11, 12].

Generally, since multiple analytes are typically treated on separate channels of the traditional SPR sensors, the resultant relatively high sample consumption and low efficiency certainly hindered their applications in many cases.As far as we know, the bio-banked collections might be rare in medical diagnosis[13, 14], as well as blood or fingerprint evidence at a crime scene[15, 16], which required to be much more critically detected. These samples that contain complex analytes are difficult to obtain since they are usually scarce or expensive. A reasonable analysis is required not only for the discovery of biomarkers potentially permitting early stage disease detection but also eventually cluing for a case[17, 18]. Although some conventional analytical techniques, e.g. high-performance liquid chromatography, mass spectrometry, fluorescence, and electrochemical sensor, have been developed for targets analysis, their shortages like complicated operation and sample preparation and poor reproducibility were still restricted in these cases[19-21]. Therefore, it is urgently needed to develop more efficient methods for simultaneous detection of multiple analytes with lower sample usages.

Some efforts have been devoted to achieve the aim using SPR biosensors, e.g., by the increase in the number of test channels and the addition of imaging systems[22-25]. Nevertheless, most of SPR instruments in the worldwide labs only possess single channel for single-target detection, which could not satisfy the demands for low-dosage and effective detection now. Hence, it is highly valuable to develop simultaneous and multiple detection on the single SPR cell. Although our previous work provided a new thought, the selection of regeneration solutions was a bit complicated[26]. It is well known that sandwich immunoassays can be used to improve the sensitivity in SPR detection[27, 28]. Due to antibodies secreted from different series have different architectures, the secondary antibodies may be specific to each right antibody[29]. Therefore, different analytes may be detected in a single run through analysis of the antibodies' binding characteristics. Based on that, we proposed to use sequential sandwich method with different antibodies to simultaneously recognize various corresponding analytes.

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DOI: 10.1039/C9AN01183H

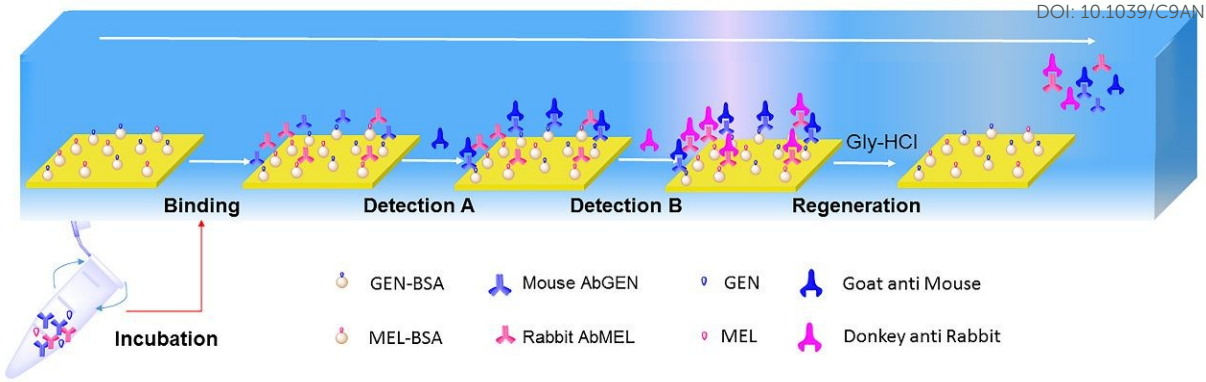

Fig. 1 Schematic diagram for the detection procedure. First, the samples were mixed with AbGEN and AbMEL for incubation. After one single sample injection, the analytes will be detected simultaneously with the sequential introduction of second antibodies in the process of detection A (AbM) and B (AbR). The last step of regeneration was performed for reuse of chips.

Considering a wide application of the developed method, the analytes should be determined in different types of samples. Due to the existence in not only food but also blood samples, gentamicin (GEN) and melamine (MEL) were selected as the model analytes in this study. And a facile sandwich immunoassay was developed for simultaneous detection of GEN and MEL in an SPR single channel. Two conjugates of antigen and bovine serum albumin (BSA), e.g. GEN-BSA and MEL-BSA, were covalently immobilized on a sensor chip. Based on the immune inhibition format, two antibodies against GEN (AbGEN, secreted by mouse) and MEL (AbMEL, secreted by rabbit) bound to the functioned chip. With a sequential injection of different secondary antibodies, GEN and MEL were simultaneously detected using a low-dosage sample (50 µL) in a single channel. Furthermore, recovery tests were also performed by spiking milk and serum with GEN and MEL, to demonstrate the analytical performance of this method.

Experimental

Materials and Reagents

GEN, MEL and glycine (Gly) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Tianjin Chemical Reagent Factory. Acetate buffers (pH 4.5), 2-(4-(2-Hydroxyethyl)-1-piperazinyl)ethanesulfonic acid-buffered saline (HBS) buffer, ethanolamine (EA), N-Hydroxysuccinimide (NHS) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from General Electric Co. (Uppsala, Sweden). GEN-BSA conjugate (GEN-BSA), MEL-BSA conjugate (MEL-BSA), the mouse antibody against GEN (AbGEN), the rabbit antibody against MEL (AbMEL), and the secondary antibodies against mouse AbGEN and rabbit AbMEL (AbM and AbR, respectively) were obtained from Huaan Magnech Bio-Tech Co. (Beijing, China). Ultrapure water obtained from a Millipore Direct-Q ultrapure water system (Boston, MA, USA) was used to prepare GEN and MEL solutions (1.0 mg/mL each, store at 4 °C). Normal human serum was obtained from Tongtuo of Chromatography Technology Co. Ltd. (Tianjin, China). Milk was purchased from a local market. All samples were processed by centrifugation (Sigma, 3-30K) using ultrafine filters (Sartorius, Germany).

Sensor platform preparation

CM5 sensor chips (GE Healthcare (Uppsala, Sweden)) containing carboxymethylated dextran matrices on gold surfaces were used as substrates for modification. GEN-BSA and MEL-BSA conjugates were immobilized on the chips using EDC/NHS method for further application. Briefly, 0.4 mol/L EDC and 0.1 mol/L NHS were mixed at a final volume of 170 µL in a 1:1 ratio, and the mixture was introduced into the channel at a flow rate of 30 µL/min. Next, GEN-BSA and MEL-BSA (2.0×10^4 ng/mL each) in acetate buffer, pH 4.5, were successively injected into the channel (5 µL/min) for approximately 300 s for immobilization. Finally, any unreacted sites were deactivated by using 126 µL of EA solution (pH 8.5) at a rate of 10 µL/min on the sensor surfaces.

Regeneration optimization

To obtain a reasonable regeneration solution, Gly-HCl solution at pH 1.2, 50 mM NaOH solution and 0.1 M HCl solution were used to remove the antibodies on the sensor surface. Here is the simple procedure. AbGEN or AbMEL was introduced into the cell to interact with GEN-BSA or MEL-BSA for 60 s. Then, regeneration solutions were injected for 30 s to elute the antibody from sensor surfaces. And results were obtained and evaluated using GE software.

Assay procedure

SPR experiments were performed on a commercial SPR device named as Biacore X100 (GE Healthcare). During experiments, the temperature of this system was maintained at 25 °C. HBS buffer (pH 7.4) was used as running buffer at a rate of 30 µL/min. Based on the immune inhibition format, GEN-BSA and MEL-BSA were immobilized on a sensor chip. Notably, test procedures were divided into two steps in the assay. As shown in the Fig. 1, AbGEN and AbMEL were firstly injected into the channel to bind with the right antigens. Before being injected, AbGEN and AbMEL (both concentrations are fixed at 1.0×10^4 ng/mL) were mixed with different concentrations of GEN and MEL standard. In the mixtures, the final concentrations of GEN standards were fixed to 0, 10, 20, 30, 40, 50, 60 ng/mL, while

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those of MEL standards were 0, 1, 5, 10, 50, 100, 200 ng/mL. 50 µL mixtures were incubated for approximately 1 min and then introduced into the cell. The free AbGEN and AbMEL in the mixtures could bind to GEN-BSA and MEL-BSA respectively on the sensor chip. Second, the secondary antibodies were sequentially injected into the channel. Briefly, 50 µL AbM (1.0×10^4 ng/mL) was introduced and specifically interacted with AbGEN; followed by the injection of AbR (2.0×10^4 ng/mL) for binding with AbMEL. The results were collected and fitted to standard curves for GEN and MEL. Ultimately, the concentrations of GEN and MEL were determined in a single run with low sample consumption and high sensitivity. In addition, the chips can be reused after regenerating with Gly–HCl (pH 1.2) for approximately 30 s. For ensuring the test validity, each cycle was performed in triplicate.

Spiking tests

For spiking tests, 50 µL serum was spiked with GEN and MEL. Then, the sample was diluted 10 times with HBS buffer for ultrafiltration-centrifugation. The ultrafiltrate was collected and taken 50 µL for detection. In the spiking samples, the final concentrations of GEN and MEL were 20, 30, 40, 50 ng/mL and 5, 10, 50, 100 ng/mL. All the tests were performed at least three times for precise results. Similar procedure was performed for the spiking test of milk.

Results and discussion

Modification of conjugates on the chip surface

For ensuring that GEN-BSA and MEL-BSA were preferably immobilized on sensor chips, acetate buffer (pH 4.5) was used to prepare the GEN-BSA and MEL-BSA solutions. At pH 4.5, BSA carries positive charges since its pI value is 4.7, while the chip surface coated with carboxymethylated dextran (pKa 3.5) exhibits negative charges[30]. Based on electrostatic interactions, BSA can be easily bound to the chip surface. Furthermore, the EDC/NHS method was performed to activate the carboxyl groups on the surface, which contributed for stably binding with BSA in a covalent bond[31].

Regeneration analysis

Regeneration analysis is a necessary step for the reuse of chips in the assay[32]. Antibodies, which served as analytes binding to antigens on the surface, should be eluted absolutely without damaging the functional surface on the chips[33]. As shown in Fig. 2 and S1, the shift in sample response and baseline was compared using three regeneration solutions: 50 mM NaOH, 0.1 M HCl and 10 mM Gly–HCl (pH 1.2). After rinsing the NaOH solution, both sample response and baseline have been changed obviously. As for GEN, the baseline of GEN dropped near 100 RU. Although AbGEN was eluted, it may affect the stability of the functional surface. In contrast to GEN, the baseline increased about 70 RU, indicating that AbMEL was not completely dissociated from the chip. And the AbMEL residue leaded to the decrease in sample response. On the other hand, using 0.1 M HCl and Gly–HCl (pH 1.2), the results of sample response and baseline tended to be stable after two reuse cycles. Since a little higher fluctuation of HCl presented on both

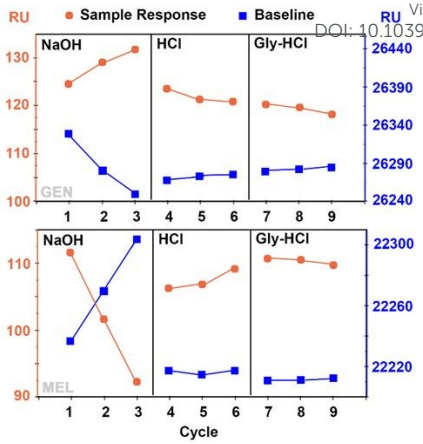

Fig. 2 The regeneration of 50 mM NaOH, 0.1 M HCl and pH 1.2 Gly-HCl solutions for GEN (up) and MEL (down).

GEN and MEL, the Gly-HCl solution was selected for regeneration in this work.

Simultaneous detection

SPR sensor possesses an excellent performance to determine molecular interactions with high sensitivity. Nevertheless, the development of multiple detection in a single channel is still worthy of study. Hence, a sequential sandwich immunoassay was attempted in this work.

First, GEN and MEL were separately tested for the examination area. To guarantee an effective binding between the antibody and antigen, an adequate amount of AbGEN solution was incubated with a GEN sample with different concentrations. Subsequently, the AbGEN/GEN mixture was introduced; followed by the injection of AbM solution. As shown in Fig. 3A, a real-time SPR sensorgram was presented for changes in sample response. It is easy to see the sample response dropped with the increase of GEN concentration. Specially, this phenomenon was attributed to AbGEN amount that bound to GEN-BSA on the surface. Since AbGEN concentration was constant and part of AbGEN interacted with GEN during incubation, the free AbGEN reciprocally depended on the GEN concentration. At the second injection, AbM was specifically bound on the AbGEN surface, and a positive relationship was obtained with the response of AbM and AbGEN. Thus, the AbM response also decreased with the increase of GEN concentration from 10 to 60 ng/mL. Moreover, a similar procedure was operated for MEL detection. As shown in Fig. 3B,

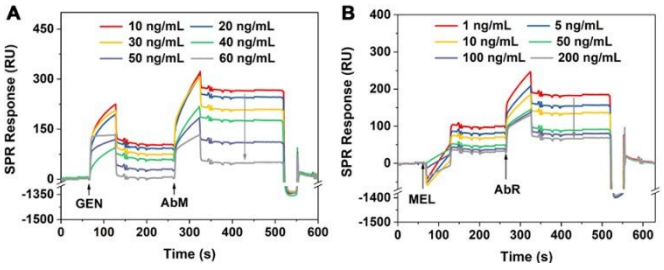

Fig. 3 SPR spectrogram for monitoring response shifts of AbGEN and AbM corresponding to the different GEN concentrations (A), and response shifts of AbMEL and AbR corresponding to the different MEL concentrations (B)

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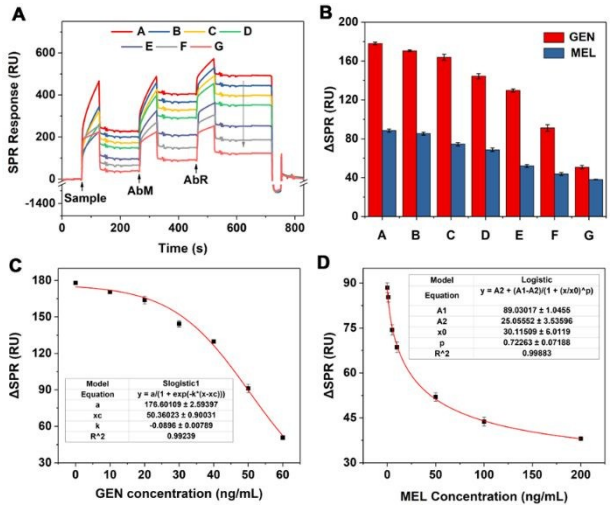

Fig. 4 SPR spectrogram for monitoring response shifts of samples, AbM and AbR. A, B, C, D, E, F, G in the figure means seven sample concentrations (A: 0 ng/mL of GEN and MEL; B: 10 ng/mL of GEN and 1 ng/mL of MEL; C: 20 ng/mL of GEN and 5 ng/mL of MEL; D: 30 ng/mL of GEN and 10 ng/mL of MEL; E: 40 ng/mL of GEN and 50 ng/mL of MEL; F: 50 ng/mL of GEN and 100 ng/mL of MEL; G: 60 ng/mL of GEN and 200 ng/mL of MEL); and the standard curves of GEN (C) and MEL (D) based on the response shifts of AbM and AbR.

an expected result was observed after injections. AbMEL response decreased with the increase in MEL concentration (from 1 to 200 ng/mL), as well as AbR response. Above all, the concentration ranges of GEN and MEL were obtained in the tests. Although some slight spikes were caused by the injection of samples, the SPR sensorgrams tended to be flat and there was almost no effect on the response results.

In the simultaneous detection, GEN, AbGEN, MEL, and AbMEL were mixed together, as mentioned above, to be injected into the sensor; followed by AbM and AbR in sequence. AbM has a specific binding with AbGEN, while AbR specifically interacts with AbMEL. Besides, to make sure the accuracy of the results, the crosstalk effects of antibodies were considered in the measurements, which showed almost no interference on each antibody-antigen interaction (Fig. S2 and Fig. S3). Therefore, based on the responses of AbM and AbR, the concentrations of GEN and MEL could be determined. As shown in Fig. 4A, different concentrations of mixtures were introduced into the sensor causing different SPR responses. The response shift had a clear increase, inversely corresponding to the concentrations of GEN and MEL. Even so, GEN and MEL could not be picked up in the mixture or in a real system. After the injection of AbM, it would bind to AbGEN leading to a specific response, which presented a similar tendency as it mentioned in the single detection. And the response shifts (ΔSPR) were collected in Fig. 4B. Here, the shift in AbM response was calculated using AbM response to minus the corresponding AbGEN response. According to the results, a standard curve was fitted well between GEN concentrations and shifts in AbM response (Fig. 4C). And the limit of detection (LOD) was 4.4 ng/mL. Based on the standard curve, an accurate concentration of GEN can be calculated by the corresponding SPR response. Then, AbR was used to recognize AbMEL for a specific response, which contributed to determine the MEL concentration in the mixture. Similar with GEN, a standard curve for MEL was obtained between shifts in AbR response and MEL concentrations with a high relationship (Fig. 4D). And its LOD was 1.3 ng/mL.

Compared with traditional detections, this method has many advantages of low-dosage sample, detection efficiency and time savings. Generally, one injection of samples can just show one target result on common sensors (single test channel). If much more targets need to be monitored, more samples will be consumed. However, the samples are rare, precious or unobtainable in some cases, such as medical discovery or criminal scene evidence. All samples should be used abstemiously and carefully in an examination. Besides, each sample has its own chip. It means that the chips should be switched and primed after finishing one test. These processes are really time consuming. In this work, GEN and MEL in samples can be simultaneously detected using a single injection of sample, together with introduction of their corresponding second antibodies (AbM and AbR).

Application in milk and serum

To determine the method feasibility in real samples, recovery studies were examined using the simultaneous detection method in milk and serum. The complex matrixes in samples

Table 1 Recovery studies for milk and serum with three concentrations of CAP and GEN by the multi–detection method

Target sample	Spiked concentration (ng/mL)	Average ± SD (ng/mL)		Recovery (%)	
		milk	serum	milk	serum
GEN	20.0	22.1 ± 0.9	21.4 ± 1.6	110.5	107.0
	30.0	30.1 ± 0.9	30.8 ± 0.4	100.4	102.7
	40.0	39.8 ± 0.7	39.5 ± 0.3	99.5	98.8
	50.0	51.8 ± 0.7	51.3 ± 1.0	103.6	102.6
MEL	5.0	5.9 ± 0.1	5.3 ± 0.7	118.0	106.0
	10.0	9.3 ± 0.5	9.3 ± 0.5	93.0	93.0
	50.0	47.9 ± 4.4	43.1 ± 1.4	95.8	86.2
	100.0	81.6 ± 8.7	91.6 ± 4.6	81.6	91.6

may adsorb non-specifically on the surface and hinder the specific interaction between antibodies and antigens[34]. Hence, the sample preparation should be considered in the tests. Additionally, we assumed this method was applied in a real case, during that samples could only be collected in a small amount. To prepare injection samples, the procedure dilution should be operated firstly, but excessive dilution may not be measured by traditional instruments. Therefore, the sample injection times should be reduced to lower the sample dilution times. After spiked with different concentrations of GEN and MEL in the milk, the samples were suitably diluted, and ultrafiltration–centrifugated to reduce the interference as we reported before. As shown in Table 1, the recovery results of GEN and MEL in milk were 99.5% - 110.5% and 81.6% – 118.0%, respectively, as well as 98.8% - 107.0% for GEN and 86.2% - 106.0% for MEL in serum.

Although some deviations or errors existed, they can be ascribed to accepted reasons. During sample injection, instrument noise may accompany resulting in the fluctuation of sensor response. Moreover, the standard curves were fitted and applied with a little deviation. Although samples had been prepared carefully, matrix effects might have a bit effect on the results. To reduce the errors, each test was performed repeatedly to get an average value. Fortunately, these errors did not affect the potential applications of this method in trace sample detections.

Conclusions

In this work, a facile method was applied for simultaneous detection of GEN and MEL using single-channel SPR sensor. With one injection, both analytes can be detected rapidly (~ 10 min) with high efficiency and sensitivity. Meanwhile, milk and serum were spiked with GEN and MEL, and examined with satisfactory recovery results (81.6% – 118.0%). All results suggested good feasibility for multiple detections in a traditional sensor. Moreover, the analytes can be multiplied in multiple-channel instrument. With the development of antibody and aptamer, this method provided a widely potential application in multiple detections.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Nos. 21621004 and 51473115) and the Natural Science Foundation of Tianjin (No. 16JCZDJC37900).

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Table of contents

A efficient and facile method of sequential sandwich immunoassay was developed for simultaneous detection in trace samples using single-channel SPR with low-dosage sample and testing time.