

# Surface plasmon resonance-based immunosensor with oriented immobilized antibody fragments on a mixed self-assembled monolayer for the determination of staphylococcal enterotoxin B

Wen-Chi Tsai · Pi-Ju Rini Pai

Received: 19 January 2009 / Accepted: 28 April 2009 / Published online: 14 May 2009  
© Springer-Verlag 2009

**Abstract** An immunosensor based on surface plasmon resonance (SPR) with a mixed self-assembled monolayer (SAM) was developed to determine staphylococcal enterotoxin B (SEB). The SAM on a gold surface was fabricated by adsorbing a mixture of 16-mercapto-1-hexadecanoic acid (16-MHA) and hexanethiol at various molar ratios. Initially, full-length anti-SEB was randomly immobilized onto the SAM to form the immunosensing surface. Through optimization of surface functionalization and anti-SEB immobilization, the SPR sensors can be applied to the determination of SEB in a linear range of 0.01–1.0  $\mu\text{g}\cdot\text{mL}^{-1}$ . Furthermore, a smaller antibody fragment (F(ab)') was generated and immobilized randomly (via amino groups) or in an oriented manner (via –SH groups) to form the immunosensing surface. The oriented immobilization of F(ab)' led to a 50% increase in the antigen binding efficiency compared to randomly immobilized covalent F(ab') fragments. The resulting calibration curve showed higher sensitivity. In addition, the specificity and applicability of the proposed immunosensor to milk samples were also demonstrated. Furthermore, the sensor can be regenerated using 0.1 M HCl, and 70% of the initial response was maintained over 3 cycles.

**Keywords** Surface plasmon resonance · Mixed self-assembled monolayers · Antibody fragments · Staphylococcal enterotoxin B · Immunosensor

## Introduction

Staphylococcal enterotoxin B (SEB) is one of the many toxins produced by *Staphylococcus aureus*. *Staphylococcus aureus* makes 12 structurally similar enterotoxins, namely, enterotoxins A, B, C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, D, and E, plus the recently discovered G, H, I, J, and K [1, 2]. These enterotoxins are heat-stable, so they can only be damaged when cooked for more than 2 h at 100°C; therefore, they can still lead to illness even after being cooked. SEB was the subject of this research. It is a 28.4-kDa polypeptide with two tightly packed domains that can resist proteases and thus, can survive in the gastrointestinal tract. When ingesting as little as 100 ng of SEB orally, patients will usually have symptoms of classic food poisoning of vomiting, diarrhea, and fever; ingestion of larger amounts, in severe cases, can lead to lethal shock [3, 4]. Not only does this toxin cause food poisoning, it is also considered a potential biological weapon and is classified as a category B select agent by the National Institutes of Health and Centers for Disease Control in the United States [4]. If exposed to SEB-contaminated air, one would have symptoms such as fever, chills, headaches, and cough. Due to its high disease-causing rate and infective ability, releasing SEB into food, water, or air on purpose would be a great danger to a nation. Thus, creating a fast, sensitive, and specific method to detect SEB in food, water, blood, urine, or tissues is required.

Immunosensors have attracted great interest in recent years for monitoring biological and chemical agents in a variety of applications. A number of different signal-transduction platforms have been designed including amperometric immunosensing [5–7], impedance spectroscopy [8, 9], QCM-based immunosensors [10–12], and surface plasmon resonance (SPR)-based immunosensors

W.-C. Tsai (✉) · P.-J. R. Pai  
Graduate Institute of Biotechnology, Chinese Culture University,  
55 Hwa-Kang Rd.,  
Taipei, Taiwan  
e-mail: wetsai@faculty.pccu.edu.tw

[13, 14]. Among these, SPR-based sensors are known to possess various technical advantages. For example, they detect biomolecular interactions without the use of labeled molecules, determine kinetics and equilibrium constants, provide real-time analysis, require low sample amounts, and have high sensitivity, specificity, and reusability [15–17]. In this particular research, we attempted to develop an SPR-based immunosensor.

SPR is a physical process which occurs when light hits a metal at a special angle under total internal reflection conditions. The angle of incidence at which SPR occurs is called the SPR angle. At the SPR angle, a sharp decrease in intensity is found. The position of the SPR angle depends on the refractive index of the sensing surface. Upon binding of macromolecules to the surface, the refractive index of the sensor surface changes, the SPR wave will change, and therefore the angle changes accordingly. As a result, the SPR angle is directly proportional to the number of molecules on top of the gold surface, and SPR angle shifts (in millidegrees) can be used as a response unit to quantify the binding of macromolecules to the sensor surface [17–19].

Successful binding of large biomolecules such as antibodies may require spacing of functional groups to prevent steric hindrance. Mixed alkanethiol monolayers provide a powerful methodology to create tailored, functional surfaces that can be applied to almost limitless research possibilities. Mixed self-assembled monolayers (SAMs) are generally constituted of one thiolate with a functional head group (such as carboxylic acid) at a low mole fraction and of another “diluting” thiolate at a high molar fraction [20]. The second thiol (the “diluting” thiolate) reduces the surface concentration of functional groups and thus minimizes steric hindrance and non-specific interactions that can produce interference signals [21]. In this study, mixed SAMs were incorporated as a method of antibody immobilization with the intention of creating a spacious environment in order to prevent overlapping of the antigen binding sites. Thiol mixtures of different molar ratios of 16-mercapto-1-hexadecanoic acid (16-MHA) to 11-mercapto-1-undecanol (11-MUOH) and 6-mercapto-1-hexanol (6-MHOH) were examined.

Furthermore, smaller and well-oriented antibody fragments were used in an attempt to further minimize the steric hindrance and increase the antigen-binding efficiency in a single step. Since sensitivity is also strongly dependent on the antigen-binding site directions, the effect of the orientation of the antigen-binding site with respect to the surface was evaluated. Several strategies have been reported to attach antibodies to surfaces in an oriented manner [22, 23] to improve the antigen-binding efficiency of the immobilized antibodies. Antibodies can be oriented on a surface by binding of the Fc region of the antibody to a layer of protein A or G [24, 25]. Methods that utilize

fragmented immunoglobulin are also available for controlled immobilization. For example, F(ab')<sub>2</sub> binding through the free sulphhydryl group opposite antigen-binding sites is a popular approach [26, 27]. In this study, we compared three different antibody immobilization strategies, all based on the binding of antibodies (or antibody fragments) to mixed SAM-coated surfaces. The three types were random immunoglobulin G (IgG), random F(ab')<sub>2</sub> fragments, and oriented F(ab')<sub>2</sub> fragments. We compared the surface densities and antigen binding efficiencies of these preparations. The applicability of the fabricated immunosensor to an actual sample was examined as well.

## Experimental

### Materials

SEB from *S. aureus*, an anti-SEB IgG developed in a rabbit, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), glycine, and phosphate-buffered saline (PBS) were obtained from Sigma ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)). 16-Mercapto-1-hexadecanoic acid (16-MHA), 11-mercapto-1-undecanol (11-MUOH), and 6-mercapto-1-hexanol (6-MHOH) were obtained from Aldrich Chemical ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)). N-Hydroxysuccinimide (NHS) was purchased from Fluka ([www.sigmaaldrich.com](http://www.sigmaaldrich.com)). The ImmunoPure<sup>®</sup> F(ab')<sub>2</sub> preparation kit was purchased from Pierce ([www.piercenet.com](http://www.piercenet.com)). Centriplus (YM-30) was obtained from Millipore ([www.millipore.com](http://www.millipore.com)). 2-(2-Pyridinyldithio)ethaneamine hydrochloride (PDEA) was purchased from Biacore ([www.biacore.com](http://www.biacore.com)). A 30% acrylamide/bis solution, 29:1, ammonium persulfate (APS), N,N,N',N'-tetramethyl-ethylenediamine (TEMED), and precision plus protein standards were obtained from BIO-RAD ([www.bio-rad.com](http://www.bio-rad.com)). Tris-HCl was purchased from Merck ([www.merck-chemicals.com](http://www.merck-chemicals.com)). Other chemicals used were of analytical grade.

### Apparatus

The SPR measurements were conducted using a single-channel AUTOLAB ESPR ([www.ecochemie.nl](http://www.ecochemie.nl)), which was operated at a constant temperature of 37°C. The geometrical setup of the instrument was in a Kretschmann configuration, and the detection was in the angular interrogation mode. It works with a laser diode fixed at a wavelength of 670 nm, using a vibrating mirror to modulate the angle of incidence of the *p*-polarized light beam on the SPR substrate. The instrument is equipped with a cuvette. A gold sensor disk (17 mm in diameter) mounted on a hemi-cylindrical lens through index-matching oil forms the

base of the cuvette. A syringe pump and a peristaltic pump perform all liquid handling. The syringe pump is used for sample mixing in the cuvette and for sample dispensing. This experimental arrangement maintains a homogeneous solution and hydrodynamic conditions. The peristaltic pump is used for draining the cuvette, with the waste going into a waste flask. The SPR angle shift (millidegree change;  $\Delta\theta$ ) was measured at a non-flow liquid condition, i.e., with the circulating pump paused. The degree of substance immobilization is given by the change in millidegrees ( $m^\circ$ ). Every 120  $m^\circ$  angle shift is equivalent to 1  $ng.mm^{-2}$ .

#### Preparation and characterization of F(ab')<sub>2</sub> fragments [28]

Anti-SEB F(ab')<sub>2</sub> fragments were prepared using an ImmunoPure<sup>®</sup> F(ab')<sub>2</sub> preparation kit. IgG of anti-SEB (10  $mg.mL^{-1}$ ) were digested with 0.125 mL of immobilized pepsin for 4 h in a shaking water bath at 37°C. The crude digests were purified with an immobilized protein A column, and F(ab')<sub>2</sub> fragments were collected. The collected samples were characterized using 12.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and concentrated with Millipore YM-30 centriplus. The final concentration was determined by a spectrophotometer at a wavelength of 280 nm.

#### Preparation and characterization of F(ab') fragments [28]

F(ab') fragments were prepared by reducing the F(ab')<sub>2</sub> fragments with 0.1 M dithiothreitol solution. The reduced samples were run through a Sephadex G-25 column and were then characterized using 12.5% SDS-PAGE and concentrated with Millipore YM-30 centriplus. The final concentration was determined by a spectrophotometer at a wavelength of 280 nm.

#### Preparation of mixed SAMs on gold surfaces [29, 30]

Prior to use, bare gold disks were completely soaked in a freshly prepared piranha solution (a 3:1 mixture of H<sub>2</sub>SO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub>) 3 times, each for 10 s, and then the disks were thoroughly washed with deionized water. A cleaned bare gold disk was then mounted onto the SPR cuvette. Thiols with carboxyl (16-MHA) and hydroxyl (6-MHOH or 11-MUOH) terminal groups were used to modify the gold disks. 16-MHA served as a linker, while 6-MHOH and 11-MUOH served as spacers.

First of all, mixed SAMs with different spacer lengths were prepared: 1 mM of 16-MHA was mixed separately with 1 mM 6-MHOH and 11-MUOH in a 1:10 ratio. Second, mixed SAMs with different concentrations were formed: 1 and 10 mM 16-MHA/6-MHOH SAMs were mixed in a 1:10 ratio. Finally, mixed SAMs with different

linker to spacer ratios were examined: 10 mM 16-MHA/6-MHOH at 1:0, 1:3, 1:10, and 1:20 ratios. Each of these SAMs was formed by depositing the thiol solution on the gold surface for 1 h. These thiol-coated gold disks were used in the following SPR experiments.

#### Immobilization of full-length antibody or antibody fragments [31]

Two immobilization strategies, in random and oriented manners, were employed (Fig. 1). For random immobilization, the antibody and fragments were immobilized via their primary amines (i.e., lysine residues). The terminal carboxylic groups of the linkers were activated with a 10-min pulse of a 1:1 mixture of 0.4 M EDC and 0.1 M NHS, and then full-length antibody or antibody fragments were placed onto the gold surface for 15 min (Fig. 1a). After immobilization, 0.2 M glycine was used to block the remaining NHS-ester groups for 10 min.

For oriented immobilization, antibody fragments were coupled onto the linker through their thiol groups (i.e., cysteine residues) which caused the antigen-binding sites to point upwards. In an identical manner to the random immobilization, linkers of the SAM were first activated with the EDC/NHS mixture for 10 min and then modified with PDEA (100 mM in 0.1 M borate buffer at pH 8.5) for 10 min. F(ab') fragments were applied to the surface and allowed to react for 15 min (Fig. 1b). The surface was then deactivated with cysteine (167 mM in 0.1 M acetate buffer at pH 4.3, with 1 M NaCl) for 10 min.

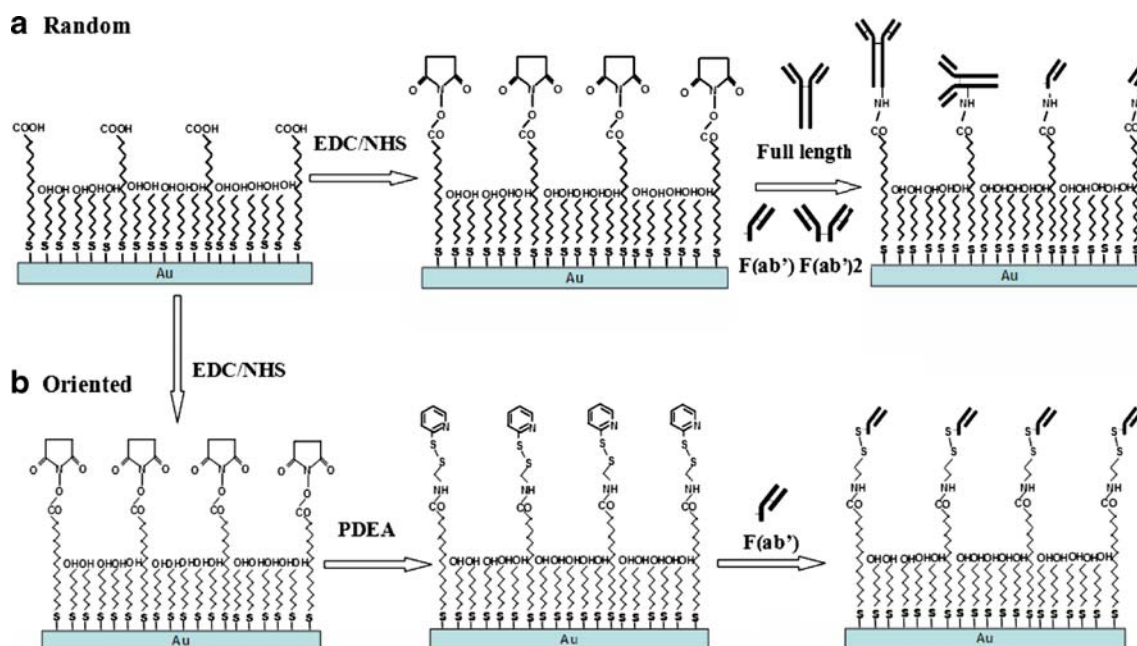
#### Detection of SEB

Phosphate-buffered saline or milk samples spiked with SEB solutions at various concentrations (10~1,000  $ng.mL^{-1}$ ) were applied to the antibody (fragment)-coated surface. After 1 h of reaction, PBS buffer was flushed over the surface to remove any loosely associated SEB. The surfaces were regenerated by applying a NaOH or HCl solution between analyte applications.

## Results and discussion

#### Functionalization of the SPR sensor disks with various mixed SAMs

It was reported that mixed SAMs can reduce the steric hindrance caused by carboxylic-terminated groups of SAMs, which results in accessibility of antibody immobilization onto the surface [21, 29–31]. For this, thiols with a short carbon chain and a small head group (11-MUOH and 6-MHOH) can be used as a spacer in conjunction with a



**Fig. 1** Schematic representation of (a) Random and (b) Orientated covalent antibody coupling procedures on mixed thiol layer

longer-chain thiol (16-MHA) with the head group of interest as a linker. To develop an optimum platform for detecting SEB, mixed SAMs were examined in terms of spacer length, thiol concentrations, and the ratio between the linker and spacer. The results showed that 10 mM mixed SAM with 16-MHA and 6-MHOH in a 1:10 ratio provided an optimum combination of reactive sites and steric hindrance of the terminal carboxylate groups (data not shown). Therefore, this protocol was used throughout the study for the surface functionalization. Optimal condition for antibody immobilization was subsequently examined, and 1 mg.mL<sup>-1</sup> of anti-SEB was considered to be the optimal concentration for surface preparation and was used throughout the study.

Standard curve obtained in PBS buffer using immobilized full-length anti-SEB

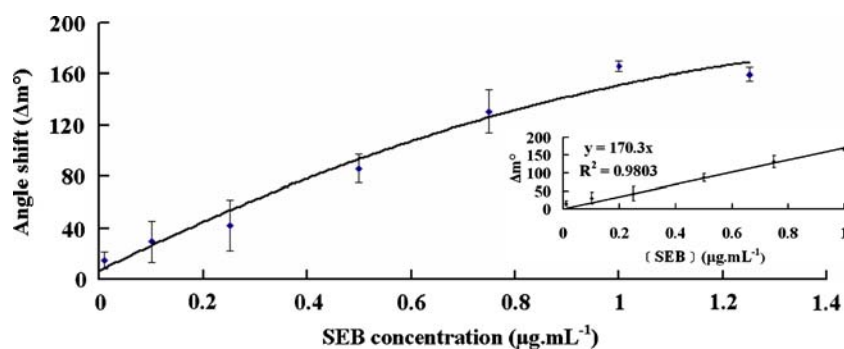
After optimizing the conditions for fabricating the immunosensor, we detected SEB in different concentrations (0.01, 0.1, 0.25, 0.5, 0.75, 1.0, and 1.25 μg.mL<sup>-1</sup>) using these immobilization conditions. The results are plotted on Fig. 2. Within the examined SEB concentration range, the responses increased accordingly and reached saturation at 1 μg.mL<sup>-1</sup> SEB. The standard curve ( $y=170.3x$ ), shown in the inset, was linear between 0.01 and 1 μg.mL<sup>-1</sup>, with  $R^2=0.98$ . These experimental results indicated that the proposed immunosensor was capable of detecting SEB at a concentration of as low as 10 ng.mL<sup>-1</sup>. This result is comparable to previous reports [32–34], which also demonstrated that immunosensors are capable of detecting SEB in the ng.mL<sup>-1</sup> range. Some of the

reports cited above were based on a sandwich format. The sandwich format amplifies the signal using a second antibody; however, this can also slow down the analysis and make real-time monitoring more complicated.

#### Antibody fragmentation and SDS-PAGE

When considering the structure of immunoglobulin, there is an unnecessary protein domain with respect to antigen binding, called the Fc domain. It was reported that by cutting off unnecessary antibody parts, the resulting antibody fragments (F(ab') and F(ab')<sub>2</sub>) can be covalently attached to the surface through the free sulfhydryl or disulfide groups on the antibody fragments [30]. Since sulfur-containing groups exist in opposition to the antigen recognition region, the processed fragments can orient to the antibody. Therefore, we prepared antibody fragments and subsequently examined the performance of the immunosurfaces prepared by the obtained fragments.

Enzymatic fragmentation of IgG for antibody fragment production was carried out using an ImunoPure® F(ab')<sub>2</sub> preparation kit (Pierce) [28, 35, 36], and the produced antibody fragments were analyzed using gel electrophoresis (Fig. 3). Lane 3 of Fig. 3 shows the presence of one major band with a molecular weight of ~100 kDa. This band can be assigned to the F(ab')<sub>2</sub> fragment. F(ab')<sub>2</sub> fragments were further reduced to F(ab') fragments using dithiothreitol as the reducing agent. Lane 4 of Fig. 3 shows that the molecular weight of the F(ab') fragment was around about 37 kDa. The molecular weight was less than expected (~50 kDa), and this could be attributed to the suboptimum



**Fig. 2** Dynamic range of staphylococcus enterotoxin B (SEB) in buffer solution obtained using an immobilized full-length anti-SEB. The self-assembled monolayer (SAM) used was 10 mM 16-MHA/6-

MHOH in a 1:10 ratio. The immobilized antibody used was 1 mg/ml anti-SEB. Vertical bars indicate the standard deviation (SD) for the mean of three measurements. The inset shows the calibration curve

reducing conditions [30]. Since the obtained F(ab') fragment retained its antigen-binding ability (data not shown), we used it with no further optimization.

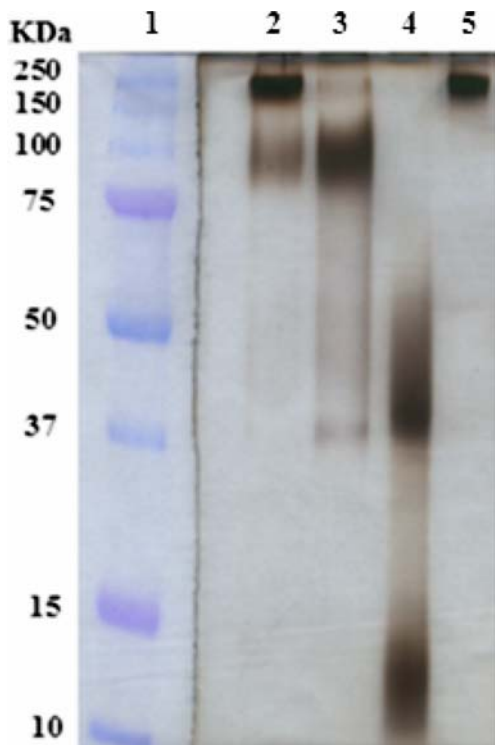
#### Comparison of immobilization of the full-length antibody and antibody fragments

Full-length IgG, F(ab')<sub>2</sub>, and F(ab') fragments were immobilized via EDC/NHS chemistry and were evaluated

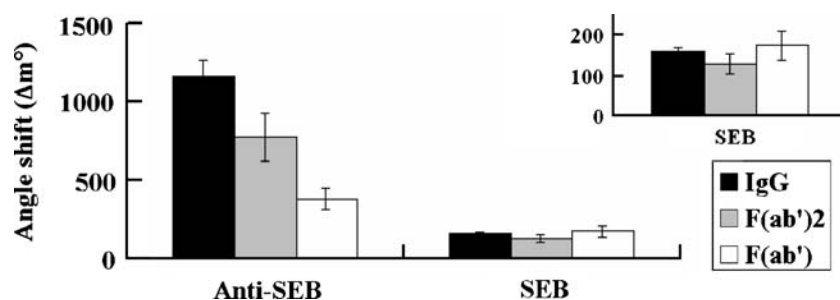
with respect to their antigen-binding response (Fig. 4). We found that absolute angle shifts caused by the immobilized molecules decreased in the order of full-length anti-SEB, F(ab')<sub>2</sub>, and F(ab'). In addition, by taking the molecular weight into account, the numbers of moles immobilized ranged from 6.4 nmol.mm<sup>-2</sup> for the full-length anti-SEB to 5.5 nmol.mm<sup>-2</sup> for the F(ab')<sub>2</sub> and 8.5 nmol.mm<sup>-2</sup> for the immobilized F(ab') fragment. It should be noted that F(ab') is univalent. The lower immobilization level of the F(ab')<sub>2</sub> and F(ab') fragments could have been a consequence of the decrease in the relative amount of available lysine residues resulting from antibody fragmentation. As far as the absolute antigen-binding response is concerned, the F(ab') fragment showed the highest antigen-binding response (175 m°), followed by the full-length IgG (162 m°) and F(ab')<sub>2</sub> fragment (129 m°). This indicates that antigen-binding sites of F(ab') fragments are more accessible than those of F(ab')<sub>2</sub> or full-length IgG. Therefore, we concluded that the reduced molecular size of F(ab') led to a further decrease in the steric hindrance and an increased sensor response. This agrees with Bonory et al.'s study [31] which examined the anti-hIgG/hIgG system and also showed a similar trend.

#### Comparison of random and oriented immobilization of F(ab') fragments on mixed SAMs

To make the antigen-binding sites point upward in an oriented position, immobilization via the free sulfhydryl group, which is structurally opposite the antigen-binding site, of the F(ab') fragment was employed. A thiol-reactive crosslinker, PDEA [37], was used in combination with EDC/NHS activation. The result of random vs. oriented immobilization is shown in Fig. 5. The amount of antibody immobilized was larger in the random fashion than in the oriented way. This may be ascribed to the high abundance of amino groups contained by a protein compared to sulfhydryl groups. Despite the fact that more F(ab') fragments were bound onto the surface in the random mode, a 26% increase in the absolute antigen-



**Fig. 3** SDS-PAGE analysis (12.5% gel) of anti-staphylococcus enterotoxin B (SEB) and anti-SEB fragments. Lane 1, Molecular weight marker; lane 2, anti-SEB after fragmentation (before protein A purification); lane 3, purified anti-SEB F(ab')<sub>2</sub> fragments; lane 4, DTT reduction of anti-SEB F(ab')<sub>2</sub> fragments to F(ab') fragments; lane 5, full-length anti-SEB. (The gel was silver stained, but the molecular weight marker was not stained.)



**Fig. 4** Effect of antibody structures on the antibody immobilization and staphylococcus enterotoxin B (SEB) determination. The self-assembled monolayer (SAM) used was 10 mM of 16-MHA/6-MHOH

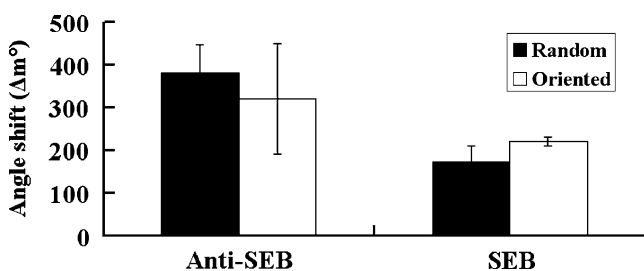
in a 1:10 ratio. On the right is an enlargement of the specific SEB angle shift. Vertical bars indicate the standard deviation (SD) for the mean of three measurements

binding signal was observed for the oriented coupling procedure (220 m°) compared to the random coupling methods (175 m°). In addition, we assessed the antigen-binding efficiency of the F(ab')-immobilized surfaces using the following equation [38]:

$$\% \text{ binding efficiency} = \frac{M_{Ab}}{M_{Ag}} \times \frac{\Delta\theta_{Ag}}{\Delta\theta_{Ab}} \times 100$$

where  $M_{Ab}$  is the molecular weight of the antibody or antibody fragments,  $\Delta\theta_{Ag}$  is the angle shift due to antigen binding,  $M_{Ag}$  is the molecular weight of the antigen, and  $\Delta\theta_{Ab}$  is the response upon antibody or fragment immobilization.

The molecular weights of F(ab') and SEB are 37 and 28.4 kDa, respectively, and the calculated antigen-binding efficiency was 60% for the random mode and 90% for the oriented mode. These calculations strongly indicate that some antigen-binding sites on the randomly immobilized F(ab') fragments were blocked due to an incorrect binding position, whereas in the oriented immobilization mode, the antigen-binding sites were more accessible to the solution. This is in agreement with previous reports [39] which claimed that F(ab')-oriented immunosurfaces result in enhanced antigen-binding activity when compared to surfaces prepared via random immobilization.



**Fig. 5** Effect of antibody immobilization orientation on the antibody immobilization and staphylococcus enterotoxin B (SEB) determination. The self-assembled monolayer (SAM) used was 10 mM of 16-MHA/6-MHOH in a 1:10 ratio. Vertical bars indicate the standard deviation (SD) for the mean of three measurements

Standard curve obtained in buffer sample using immobilized F(ab')

Based on the above studies regarding the effects of antibody structure and orientation, SEB in different concentrations (1, 0.75, 0.5, 0.25, 0.1, and 0.01  $\mu\text{g.mL}^{-1}$ ) was detected using the new immobilization condition. A standard curve has a linear relationship between 0.01 and 1  $\mu\text{g.mL}^{-1}$  ( $y=211.39x$ ,  $R^2=0.99$ ) was observed. According to a previous report [40], sensitivity is defined as the change in response that results from a unit change in concentration of an analyte,  $\Delta\text{response}/\Delta\text{concentration}$ . For the simplest case of a univariate calibration scheme, the slope of the curve is the sensitivity relative to the analyte. When we overlaid the calibration curves obtained from the full-length antibody (Fig. 2) and F(ab') fragment, we noted that the latter had a higher slope. Once more, the use of a smaller F(ab') fragment in combination with an oriented immobilization may have produced increased sensitivity.

Standard curve obtained in a milk sample using the immobilized F(ab')

Applicability of SPR immunosensor systems to precisely determine analytes of food industry interest from diverse samples is highly desirable to establish quick and reliable

**Table 1** Effect of two regeneration solutions on the reusability of the staphylococcus enterotoxin B (SEB) immunosensor

Regeneration solution	SEB		
	Trial	$\Delta\theta$ (millidegrees)	Recovery (%)
HCl (0.1 M)	1	227.7±92.0	100
	2	171.0±68.4	75.1
	3	158.9±64.0	69.8
NaOH (1.2 M)	1	220.0±71.1	100
	2	8.5±2.4	3.9
	3	7.6±5.2	3.5

analytical tools and avoid time-consuming pretreatment methods. To investigate the performance of the F(ab')-based biosensor in a complex medium, a milk solution was selected as a representative sample. Milk samples spiked with various concentrations of SEB standard solutions, to final concentrations of 1, 0.75, 0.5, 0.25, and 0.1  $\mu\text{g}\cdot\text{mL}^{-1}$ , were directly analyzed using the proposed immunosensor. A linear relationship ( $y=310.28x+337.5$ ,  $R^2=0.99$ ) was observed within the examined SEB concentration range (0.1–1.0  $\mu\text{g}\cdot\text{mL}^{-1}$ ). A clear matrix effect was observed in the absence of SEB. The difference in the refractive index between milk samples and buffer solutions as well as the non-specific adsorption of molecules from milk samples onto the sensor surface may have been responsible for this. The result demonstrated the feasibility of applying the immunosensor to a complex sample, and thus it is possible to measure the analyte directly in milk samples without dilution pretreatment.

#### Cross-reactivity

Cross-reactivity is an important analytical parameter regarding the specificity and reliability of immunoassays. Due to its close relatedness to SEB [41], SEA was selected as a model system to evaluate the specificity of the proposed immunosensor. At SEA concentrations of  $< 1 \mu\text{g}\cdot\text{mL}^{-1}$ , the response was negligible (data not shown), while at  $1 \mu\text{g}\cdot\text{mL}^{-1}$  of SEA, a  $3.3\text{-m}^\circ$  angle shift was observed. This accounts for about 1.5% of the response compared to SEB at the same concentration. This result indicates that SEA showed a small interference effect on SEB detection in the present immunoassay.

#### Regeneration

Surface regeneration for cyclic use of the same sensor platform in serial determinations of various analytical samples is an important part of SPR immunoassays. Good regeneration results in the removal of SEB from the surface, while the immobilized antibodies remain intact. Since the native tertiary structure of antibodies are sensitive to pH, a variety of regeneration buffers in the pH range from highly acidic to basic have been used for surface regeneration [42, 43]. As shown in Table 1, with 0.1 M HCl as a regeneration solution, a decrease of about 30% in the response signal was observed over 3 cycles. When using 1.2 M NaOH as the regeneration buffer, a large decrease in the signal was observed, and only 4% of the initial response was maintained. These results suggest that 1.2 M NaOH not only dissociates SEB but also causes denaturation of the antibody, thus resulting in a diminished lifetime of the immunosensor. As such, 0.1 M HCl seems to be more feasible as a regeneration buffer for the examined sensor.

## Conclusions

By controlling the orientation of the antibody molecules immobilized on the sensor surface using a mixed SAM, we developed an SPR-based immunosensor to detect SEB with satisfactory sensitivity. The optimum mixed SAM on the Au surface was fabricated by adsorbing 10 mM 16-MHA and 6-MHOH in a 1:10 ratio. The higher response signal observed was attributed to a sufficient amount of reactive sites as well as reduced steric hindrance. Smaller receptor molecules such as F(ab)' decreased the steric hindrance and thus improved accessibility for the antigen. Furthermore, it was demonstrated that specifically immobilized F(ab') fragments can be used to produce immunosurfaces with higher antigen-binding efficiencies than corresponding surfaces modified with randomly immobilized antibody fragments. Pure SEB and SEB in spiked milk were detected with satisfactory specificities. These results demonstrate that the proposed immunosensor could be a useful tool for real-time analysis of SEB in foods.

## References

- Muller-Alouf H, Carnoy C, Simonet M, Alouf JE (2001) Superantigen bacterial toxins: state of the art. *Toxicon* 39:1691
- Desouza IA, Franco-Penteado CF, Camargo EA, Lima CSP, Teixeira SA, Muscara MN, De Nucci G, Antunes E (2006) Acute pulmonary inflammation induced by exposure of the airways to staphylococcal enterotoxin type B in rats. *Toxicol Appl Pharmacol* 217:107
- Ruan C, Zeng K, Varghese OK, Grimes CA (2004) A staphylococcal enterotoxin B magnetoelastic immunosensor. *Biosens Bioelectron* 20:585
- Mantis NJ (2005) Vaccines against the category B toxins: staphylococcal enterotoxin B, epsilon toxin and ricin. *Adv Drug Del Rev* 57:1424
- Dai Z, Yan F, Yu H, Hu X, Ju H (2004) Novel amperometric immunosensor for rapid separation-free immunoassay of carcinoembryonic antigen. *J Immun Meth* 287:13
- Micheli L, Grecco R, Badea M, Moscone D, Pallechi G (2005) An electrochemical immunosensor for aflatoxin M1 determination in milk using screen-printed electrodes. *Biosens Bioelectron* 21:588
- Bojorge-Ramirez NI, Salgado AM, Valdman B (2007) Amperometric immunosensor for detecting *Schistosoma mansoni*. *Antibody. Assay Drug Develop Technol* 5:673
- Lillie G, Payne P, Vadgama P (2001) Electrochemical impedance spectroscopy as a platform for reagentless bioaffinity sensing. *Sensors Actuators B* 78:249
- Darain F, Park DS, Park JS, Shim YB (2004) Development of an immunosensor for the detection of vitellogenin using impedance spectroscopy. *Biosens Bioelectron* 19:1245
- Vaughan RD, O'Sullivan CK, Guilbault GG (2001) Development of a quartz crystal microbalance (QCM) immunosensor for the detection of *Listeria monocytogenes*. *Enzyme Microb Technol* 29:635
- Tsai WC, Lin IC (2005) Development of a piezoelectric immunosensor for the detection of alpha-fetoprotein. *Sensors Actuators B* 106:455

12. Hsieh CK, Tsai WC (2007) QCM-based immunosensor for the determination of Ochratoxin A. *Anal Lett* 40:1979
13. Oh BK, Kim YK, Park KW, Lee WH, Choi JW (2004) Surface plasmon resonance immunosensor for the detection of *Salmonella typhimurium*. *Biosens Bioelectron* 19:1497
14. Jyoung JY, Hong S, Lee W, Choi JW (2006) Immunosensor for the detection of *Vibrio cholerae* O1 using surface plasmon resonance. *Biosens Bioelectron* 21:2315
15. Green RJ, Frazier RA, Shakesheff KM, Davies MC, Roberts CJ, Tendler SJB (2000) Surface plasmon resonance analysis of dynamic biological interactions with biomaterials. *Biomaterials* 21:1823
16. Boozer C, Kim G, Cong S, Guan HW (2006) T. Londergan, Looking towards label-free biomolecular interaction analysis in a high-throughput format: a review of new surface plasmon resonance technologies. *Curr Opin Biotechnol* 17:400
17. Homola J (2006) Surface plasmon resonance based sensors. Springer, Heidelberg
18. McDonnell JM (2001) Surface plasmon resonance: towards an understanding of the mechanism of biological molecular recognition. *Curr Opin Chem Biol* 5:572
19. Ekgasit S, Tangcharoenbumrungsuk A, Yu F, Baba A, Knoll W (2005) Resonance shifts in SPR curves of nonabsorbing, weakly absorbing, and strongly absorbing dielectrics. *Sensors Actuators B* 105:532
20. Briand E, Salmain M, Herry JM, Perrot H, Compere C, Pradier CM (2006) Building of an immunosensor: How can the composition and structure of the thiol attachment layer affect the immunosensor efficiency? *Biosens Bioelectron* 22:440
21. Frederix F, Bonroy K, Laureyn W, Reekmans G, Campitelli A, Dehaen W, Maes G (2003) Enhanced performance of an affinity biosensor interface based on mixed self-assembled monolayers of thiols on gold. *Langmuir* 19:4351
22. Lu B, Smyth MR, O'Kennedy R (1996) Oriented immobilization of antibodies and its applications in immunoassays and immunosensors. *Analyst* 121:29
23. Babacan S, Pivarnik P, Letcher S, Rand AG (2000) Evaluation of antibody immobilization methods for piezoelectric biosensor application. *Biosens Bioelectron* 15:615
24. Lee W, Lee DB, Oh BK, Lee WH, Choi JW (2004) Nanoscale fabrication of protein A on self-assembled monolayer and its application to surface plasmon resonance immunosensor. *Enzyme Microb Technol* 35:678
25. Chung JW, Park JM, Bemhardt R, Pyun JC (2006) Immunosensor with a controlled orientation of antibodies by using NeutrAvidin-protein A complex at immunoaffinity layer. *J Biotech* 126:325
26. Vikholm I (2005) Self-assembly of antibody fragments and polymers onto gold for immunoweneing. *Sensors Actuators B* 106:311
27. Vikholm-Lundin I, Albers WM (2006) Site-directed immobilisation of antibody fragments for detection of C-reactive protein. *Biosens Bioelectron* 21:1141
28. Brogan KL, Wolfe KN, Jones PA, Schoenfisch MH (2003) Direct oriented immobilization of F(ab') antibody fragments on gold. *Anal Chim Acta* 496:73
29. Choi SH, Lee JW, Sim SJ (2005) Enhanced performance of a surface plasmon resonance immunosensor for detecting Ab-GAD antibody based on the modified self-assembled monolayers. *Biosens Bioelectron* 21:378
30. Lee W, Oh BK, Lee WH, Choi JW (2005) Immobilization of antibody fragment for immunosensor application based on surface plasmon resonance. *Colloids surf B: Biointerfaces* 40:143
31. Bonroy K, Frederix F, Reekmans G, Dewolf E, De Palma R, Borghs G, Declerck P, Goddeeris B (2006) Comparison of random and oriented immobilisation of antibody fragments on mixed self-assembled monolayers. *J Immun Meth* 312:167
32. Rasooly L, Rasooly A (1999) Real time biosensor analysis of staphylococcal enterotoxin A in food. *Int J Food Microbiol* 49:119
33. Rasooly A (2001) Surface plasmon resonance analysis of staphylococcal enterotoxin B in food. *J Food Protect* 64:37
34. Slavik R, Homola J, Brynda E (2002) A miniature fiber optic surface plasmon resonance sensor for fast detection of staphylococcal enterotoxin B. *Biosens Bioelectron* 17:591
35. Domen PL, Nevens JR, Mallia AK, Hermanson GT, Klenk DC (1990) Site-directed immobilization of proteins. *J Chromatogr* 510:293
36. Jones RGA, Landon J (2002) Enhanced pepsin digestion: a novel process for purifying antibody F(ab')<sub>2</sub> fragments in high yield from serum. *J Immunol Meth* 263:57
37. Frederix F, Bonroy K, Reekmans G, Laureyn E, Campitelli A, Abramov MA, Dehaen W, Maes G (2004) Reduced nonspecific adsorption on covalently immobilized protein surfaces using poly (ethylene oxide) containing blocking agents. *J Biochem Biophys Meth* 58:67
38. Brogan KL, Schoenfisch MH (2005) Influence of antibody immobilization strategy on molecular recognition force microscopy measurements. *Langmuir* 21:3054
39. O'Brien JC, Jones VW, Porter MD, Mosher CL, Henderson E (2000) Immunosensing platforms using spontaneously adsorbed antibody fragments on gold. *Anal Chem* 72:703
40. Cunningham AJ (1998) Biosensors and bioanalytical challenges. In: Cunningham AJ (ed) *Introduction to bioanalytical sensors*, 1st edn. Wiley, New York, p 35
41. Babacan N, Rasooly A (2000) Staphylococcal enterotoxins. *Int J Food Microbiol* 61:1
42. Pei R, Yang X, Wang E (2002) Enhanced surface plasmon resonance immunoassay for human complement factor 4. *Anal Chim Acta* 453:173
43. Mauriz E, Calle A, Montoya A, Lechuga LM (2006) Determination of environment organic pollutants with a portable optical immunosensor. *Talanta* 69:359