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Full Length Research Paper

The utilization of BSA-modified chip on the investigation of ligand/protein interaction with surface plasma resonance

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The kinetic behavior of glutathione (GSH)/ glutathione-S-transferase (GST) was investigated using surface plasmon resonance (SPR). Here, an alkanethiol-modified chip incorporated with bovine serum albumin (BSA) was employed. Subsequently, GSH was anchored on BSA surface only in the experimental channel and the without-active BSA surface was designed as the reference channel to improve the quality of the binding data and prevent a number of experimental artifacts to complicate the final biosensor analysis. Our results demonstrated that the BSA-modified chip was effective not only in binding the target proteins but also in suppressing the nonspecific binding (NSB) of proteins.

Key words: Surface plasmon resonance, bovine serum albumin, glutathione, glutathione-S-transferase, alkanethiol.

INTRODUCTION

Surface plasmon resonance (SPR) demonstrated its power on sensing immunoglobulin antibodies in the early 1980s (Liedberg et al., 1983). However, of recent, the potential application of surface plasmon resonance in environmental protection (Ji et al., 2004; Ligler et al., 2003), biotechnology (Ladd et al., 2008), medical diagnostics (Yonzon et al., 2004), drug screening (Yuan et al., 2008), food safety (Ligler et al., 2003; Wiskur and Anslyn, 2001) and security (Bauer et al., 2003) has been realized. However, many investigators collect data under conditions that are not suitable for measuring binding kinetics by SPR (Lipschultz et al., 2000; Myszka, 1999; Karssson and Fält, 1997; Oddie et al., 1997). The utilization of a reference surface can dramatically improve the quality of the binding data by correcting artifacts, such as bulk refractive index changes, matrix effects, nonspecific binding, injection noise and baseline drift (Myszka, 1999; Myszka, 1999a; Myszka, 1999a; Myszka, 1998; Chen et al., 2004). Up till now, a variety of methods designing the reference surface have been developed, which fall primarily into two categories: an approach for utilizing the hydrophilic surface (Sigal et al., 1998), such as the commercially available carboxymethyl dextran- coated substrates (CM5) or the mixed self-assembled monolayers (SAMs) systems. However, both have their limitation. The dextran substrates are convenient to use because a variety of chemical methods for immobilizing proteins or low-molecular-weight ligands to dextran have been developed but have disadvantages associated with nonspecific binding, exclusion of large proteins from the interior of the gel, ambiguities in the influence of mass transport on the

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Abbreviations: GSH, glutathione; **GST,** glutathione-S-transferase; **SPR,** surface plasmon resonance; **NSB,** nonspecific binding; **BSA,** bovine serum albumin; **SAMs,** self-assembled monolayers; **PDEA,** 2-(2-pyridinyldithio) ethane amine hydrochloride**; BS^3,** Bis(3-sulfo-N-hydroxysuccinimide ester) sodium salt; **GMBS,** suberic acid 4-maleimidobutyric acid N-hydroxysuccinimide ester; **PBS,** phosphate buffered saline tablets; **DMSO,** dimethylsulfoxide; **EDC,** 1-ethyl-3-[3- dimethylaminopropyl]carbodiimide hydrochloride; **NHS,** N-hydroxysuccinimide.

values of kinetic constant and high cost. The main limitation on the mixed SAMs is that the experimental channel and reference channel can not be independently modified with active materials or inert materials. In this case, the contradictions have emerged between the minimal NSB with the incense of inert-function-group and the lower response unity with the decrease of the active-function-group. Another way is to treat the reference surface with a non-interacting protein. However, its widespread use was limited due to the trouble of looking for a suitable non-interacting protein to mimic the probe protein.

BSA, a traditional blocking agent, was widely used to reduce background. In general, researcher incubated protein arrays or chips with bovine serum albumin solution (Jung et al., 2006) or conjugated the ligand with the bovine serum albumin (Dong et al., 2008; Lu et al., 2001) to inhibit nonspecific bindings. Here, the BSA-incorporated chip was introduced into the investigation on the ligand-protein interactions using SPR. Reduced glutathione (GSH), as a ligand, was anchored on the active BSA matrix and the inactive BSA surface was naturally designed as the reference channel. There are several advantages: 1) the conjugation of BSA was employed as a blocking motif as well as an active functional group provider; 2) the random distribution of amines over BSA matrices would reduce the steric effects during adsorption phenomena involving macromolecules, which is the shortcomings of the traditional CM5 chip; 3) It avoids the trouble of looking for the non-interacting protein; 4) the systematic artifacts could be substracted well enough without the contradictions that have arisen in the utilization of the mixed SAM system; 5) only the simple chemical method was involved during the total modification art with the low cost. Subsequently, the kinetic behavior of GSH/GST was investigated. The results proved that the BSA-modified chip was effective not only in binding the target protein but also in suppressing the nonspecific binding (NSB) of protein.

MATERIALS AND METHODS

General supplies and equipments

Gold wire (diameter: 1.0 mm, purity: 99.99%), titanium foil (thickness: 0.127 mm), 2-(2-pyridinyldithio) ethane amine hydrochloride (PDEA), Bis(3-sulfo-N-hydroxysuccinimide ester) sodium salt (BS^3), suberic acid 4-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS), reduced glutathione (GSH), bovine serum albumin (BSA) and phosphate buffered saline tablets (PBS) were obtained from Sigma-Aldrich. 11-amino-1-undecanethiol was purchased from Dojinodo Chemicals of Japan. Glutathione-S-transferase (GST) was prepared according to the previously described method (Chen et al., 2004). All other chemicals were of analytical reagent grade and were used without further purification. Deionized water (18 $M\Omega \cdot cm^{-1}$) was obtained by passing distilled water through a Barnstead E-pure 3-Module system. BIAcore X instrument was used for all SPR studies described here. Static contact angle was measured with a face contact angle goniometer (CA-D) from Kyowa Interface Co. The thickness was measured with a variable angle ellipsometry system (model M-44) from J.A. Woollam Co.

Preparation of GSH substrate

The alkanethiol-modifed chips were prepared according to the traditional method. Before SPR experiment, all alkanethiol-modified chips should be incubated with the running buffer for several hours in order to obtain the stable baseline. Then, the chip was inserted into BIAcore-X machine. Subsequently, a mixture of Bis(3-sulfo-N-hydroxysuccinimide) suberate sodium salt (BS^3) (3 mM), dimethylsulfoxide (DMSO), $NaHCO_3$ (100 µL, 3:7(v/v), pH = 8.5) and BSA (100 µM, 100 µL) in PBS buffer was pulsed into two channels of integrated fluidic cartridge (IFC) to activate the surface at a flow rate of 4 $\mu L min^{-1}$ under room temperature. After a stable baseline was obtained, a mixture of N-hydroxysuccinimide 4-maleimidobutyrate (GMBS) (3 mmol/L), DMSO and $NaHCO_3$ (100 µL, 3:7 (v/v), pH = 8.5) was injected only into Fc2 at 4 µL/min to activate the BSA-bounded matrix. Finally, a mixture of GSH (100 mmol/L) and PBS buffer (100 µL) was injected into Fc2 for 25 min and the 2-mercaptoethanol was introduced to block the remained active functional group. In our experiment, all buffers were sterilized, degassed and filtered before use. Simultaneously, to prevent the formation of bubbles, the cleaning step (flush or prime) was repeated at least three times after every experiment cycle. A continuous flow pump was filled with PBS buffer (pH 7.4) including 0.005 % Tween-20 (v/v).

Investigation of nonspecific binding

A freshly-prepared GSH chip was inserted into Biacore machine. Then, a mixture of GST and PBS buffer (50 µg/mL) with 0.05% Twee-20 (v/v)) was injected into both channels at 10 µL/min to collect the data at 25℃. After baseline stable, the washing buffer (GSH 20 mmol/L, Tris buffer, pH 8.0) was flashed to regenerate the sensorchip. The same procedure was repeated at least three times.

Investigation of kinetic constant

A series of GST solutions in PBS buffer with 0.05% Twee-20 (v/v) (from 12.5 µg/mL to 100 µg/mL) were prepared and injected into both channels of IFC at 10 µL/min, sequencely. After every experimental cycle, the GSH sensorchip should be regenerated with the washing buffer described as above. The subtracted sensorgrams were fitted to a sigmoid curve equation using BIAevaluation software.

Control experiment

The commercial product (CM5 chip) was utilized to investigate the interaction of GSH/GST as a control experiment. 10 µL NHS/EDC was flashed into the Fc1 of SPR. Subsequently, 20 µL PDEA in 0.1 mol/L borate buffer (PH 8.5) was introduced into SPR. Next a mixture of GSH (100 mmol/L) and PBS buffer (100 µL) was injected into Fc2 for 25 min. Finally a solution of GST (from 12.5 to 100 µg/mL) was flushed from the inactive channel (Fc1) to the active channel (Fc2), respectively. The same experimental steps were repeated with the activated/blocked surface by injecting NHS/EDC into two channels and blocking reference surface with mercaptoethanol. After every experimental cycle, the senserchip was regenerated with the washing buffer (GSH 20 mmol/L, Tris buffer, pH 8.0).

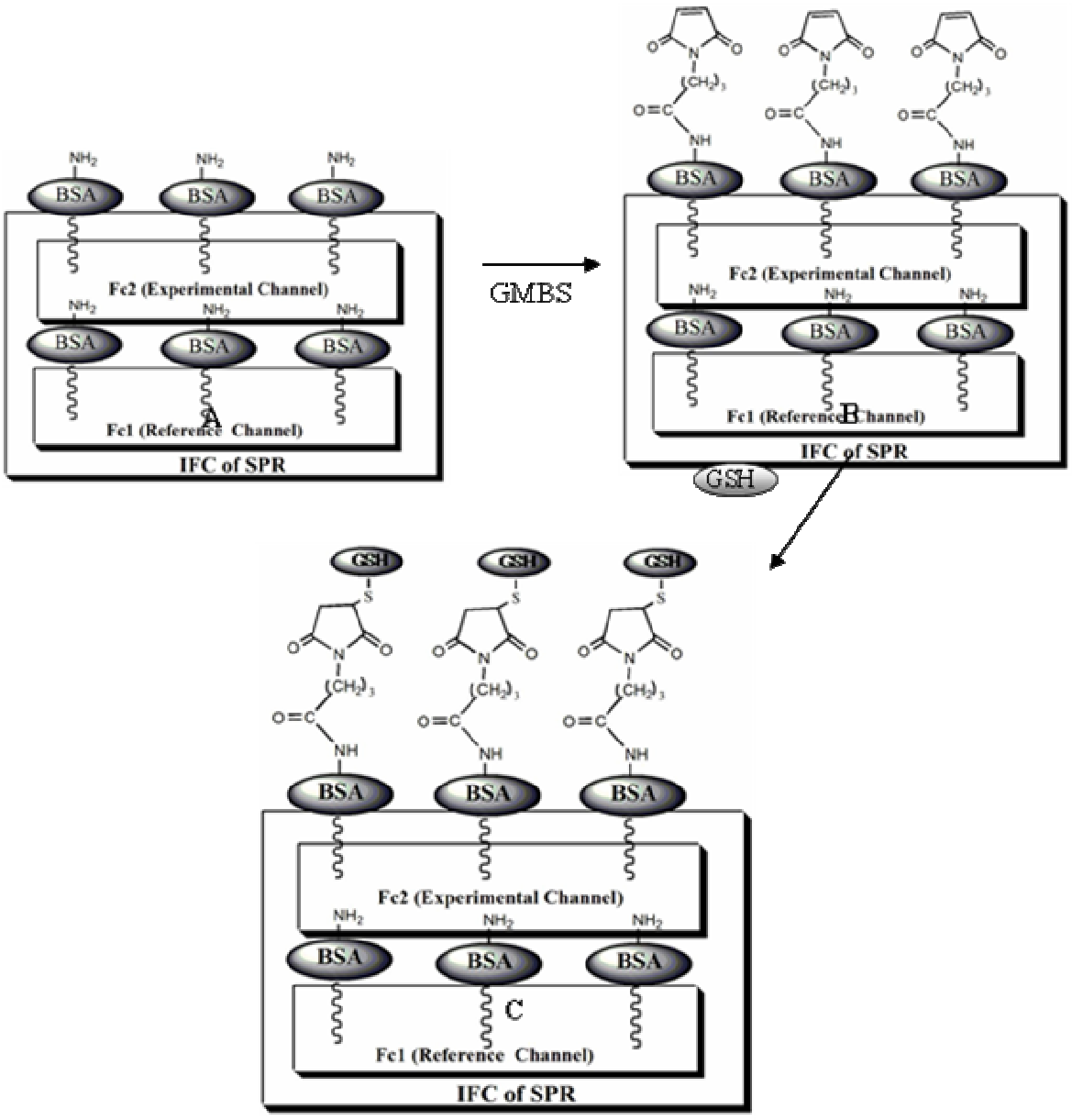

Figure 1. Schematic diagram of surface modification of BSA biochip with GSH. (**A**) BSA was modified on the alkanethiol-modified biochip with a homobifunctional linker (BS^3). (**B, C**) GSH was immobilized on the top of BSA with GMBS linker.

RESULTS

The experimental model

The substrates were prepared on glass cover slips by evaporating a thin layer of titanium (1 – 5 nm) to promote the adhesion of gold, followed by a thin film of gold (49 nm) for SPR. A layer of this thickness is sufficient to allow the total internal reflection of the laser beam used in SPR. Then, the freshly prepared gold substrates were modified with 11-amion-1-undecanethiol with an enough long spacer. Although the complete self-assembled mono-layers (SAMs) of a single alkanethiol on gold form in 12 min, we usually choose to leave gold substrates in thiol solutions for 8 h. The film thickness of 11-amino-1-undecanethiol monolayers is at least 1.6 and 2.7 nm for best, which is in good agreement with those expected for a well-packed SAM. Subsequently, bovine serum albumin (BSA) was covalently binding with the alkanethiol-modified chip with BS^3 linker. After being self-assembled with BSA, 40~57 Å increment of thickness was observed. The intact sulfonated N-hydroxysuccinimide group of BS^3 was displaced by the free amine group of chip and BSA to form a stable amide group (Figure 1A) (Chen et

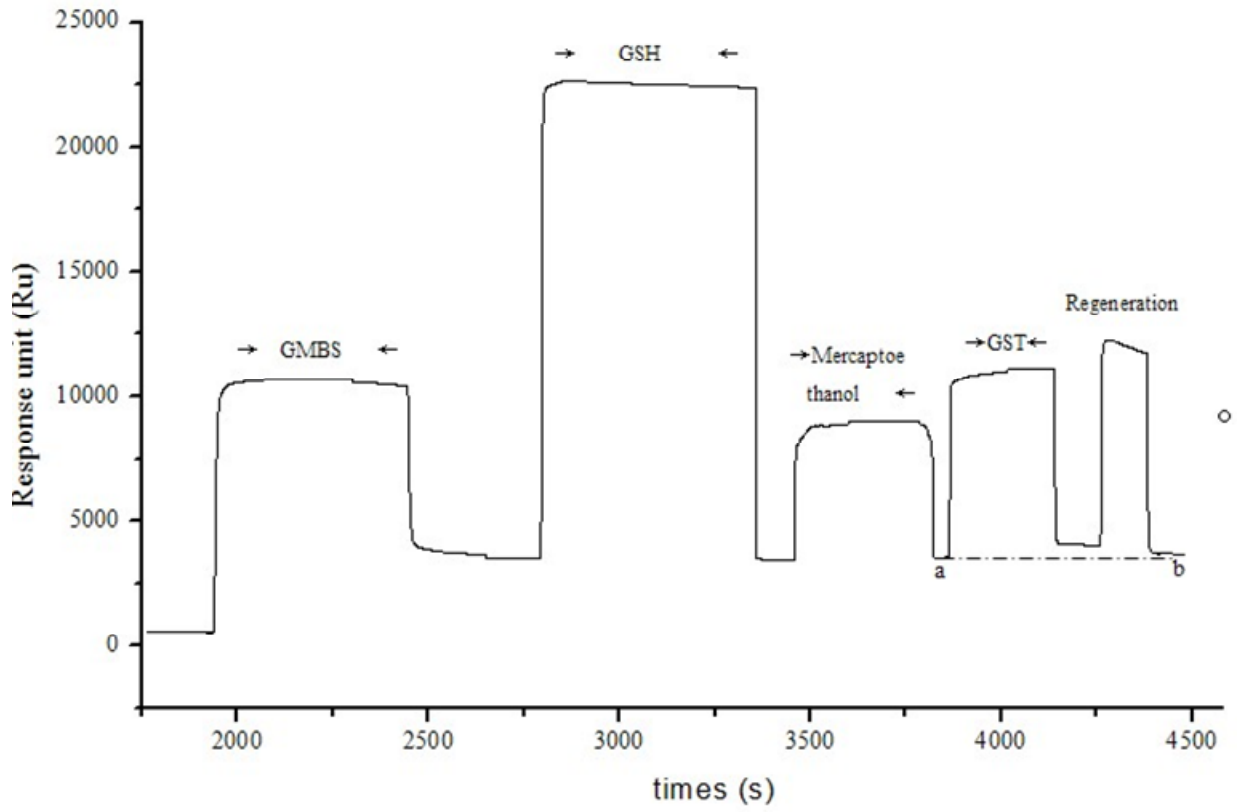

Figure 2. Sensorgram showing an entire experimental step: The modification of BSA surface with GMBS and GSH; The blocking steps with Mercaptoethanol; The injection of GST; Removal of bound species from the surface during injection of regeneration solution (20mM GSH, Tris buffer, PH 8.0). “a” represents the start point before the injection of GST, “b” the termination point after the regeneration

al., 2004).

In Biacore X machine, there are two channels, Fc1 and Fc2. After the BSA-modified chip was inserted into machine, GMBS linker was flashed only into the experimental channel (Fc2). Although GMBS, a heterobifunctional linker, is reactive with both thiol and amine group, reaction of the amine groups of BSA to release N-hydroxysuccinimide group was dominant due to low concentration of sulfhydryl group of BSA (Figure 1B). At a subsequent step, the sulfhydryl group of GSH covalently adds into the maleimido moiety of GMBS. GMBS with over 6 Å chain length allows GSH to stick out from the surface and therefore makes GSH more accessible to be ligand (Figure 1C). In order to reduce the steric hindrance resulting from the densely low-molecular-weight ligand, around 102 Ru was controlled for GSH in every experiment.

Regeneration condition

Before measurement of the kinetic behavior of GSH/GST, many prior experiments have been carried out to establish the optimized regeneration condition. In our cases, 20 mM GSH in tris buffer could rapidly and efficiently regenerate the experimental chip and an entire sensorgram was shown in Figure 2. The sensor surface contains the active ligands, ready to capture the target analytes. On injecting the solution containing the GST, they are captured on the surface. As indicated in Figure 2, the accumulated mass was obtained from the SPR response (the increment of 525.2 Ru). Finally, 20 mM GSH in tris buffer was injected, which breaks the specific binding between GST and GSH (The decrease of 508.2 Ru). Then, the ligands remain on the sensor, whereas the target analytes are quantitatively removed. “a” and “b” in Figure 3 represents the start point before the injection of GST and the termination point after the regeneration, respectively. Two points we can see were almost on a straight line. Accurately, 96.7% of matrix could be recovered for each cycle.

Nonspecific binding

In the investigation on the interaction of ligand/protein using SPR, the interruption from NSB should be addressed first. In our case, 50 µg/moL target protein was

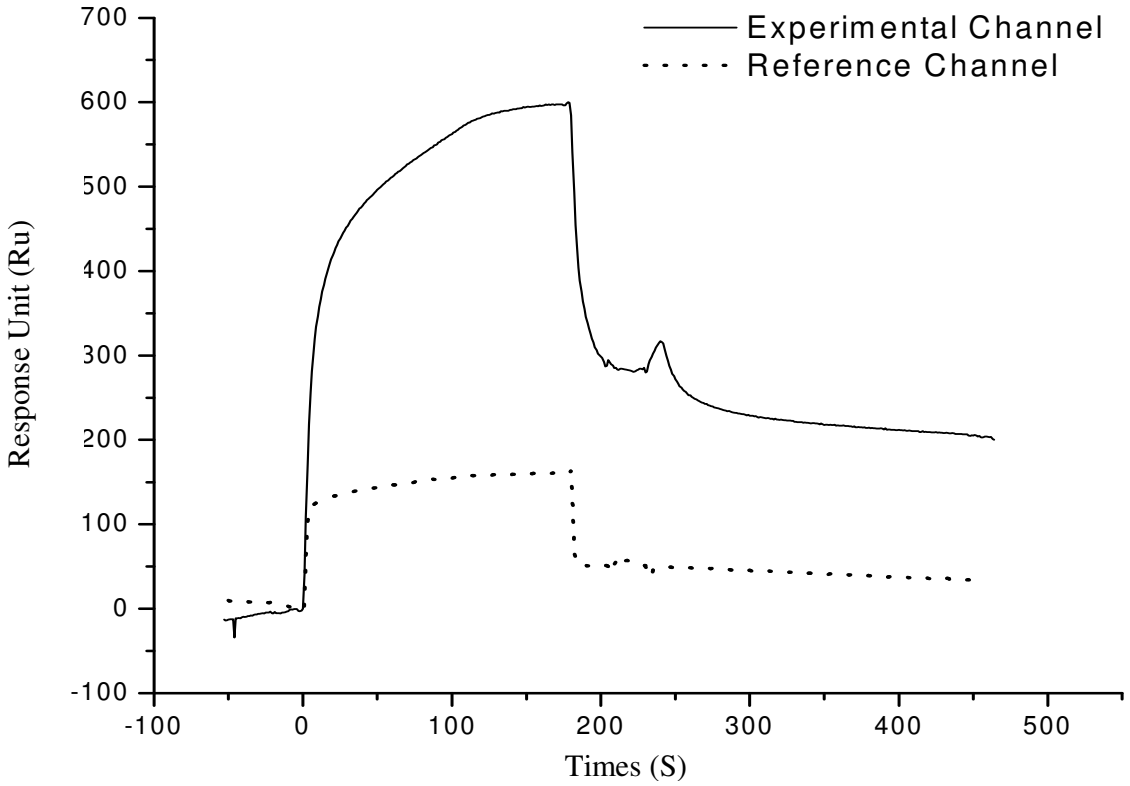

Figure 3. In the experimental channel, the real-time sensorgram of GSH/GST (50 µg/mL) binding interaction was shown with solid line. In the reference channel, dot line was used.

injected into the active/inactive BSA surface simultaneously to detect the NSB. The response unit obtained from the experimental channel (Fc2) and the reference one (Fc1) was showed in Figure 3. 264 Ru was observed for Fc2 and 18 Ru for Fc1. The change in SPR signal, termed the SPR response presented in resonance units (RU), is directly related to the change in surface concentration of biomolecules. An SPR response of 1,000 RU is equivalent to the change in protein surface concentration of 1 ng/mm^2. Therefore the surface densities of two samples were 0.264 and 0.0018 ng/mm^2, respectively. The signal from the experiment channel was 14.7 times larger than that of reference channel. In other words, it exhibits low non-specific binding (aground 6%) and indicated that the binding of GSH/GST was effective without the interruption from NBS.

Kinetic constant

After those prior experiments were finished, the kinetic constant of GSH/GST was investigated using SPR (BIAcore X). By changing the concentration of GST, binding constant and kinetic constant of GSH/GST could be easily calculated with BIAevaluation software. Therefore, a series of concentration of GST (from 12.5~100 µg/mL) was prepared. For the collected data, signal changes on the unactivated control channel have been subtracted using in-line reference. Then, the subtracted sensorgrams were fitted to a sigmoid curve equation using system software (Figure 4). Since GST has only a single binding cleft per one subunit, curves generated with serial analyte concentrations were applied globally to the 1:1 Langmuir binding model with correction for baseline drifting. The equilibrium dissociation constant values (KD = 10^6) evaluated by Biacore X for GSH/GST showed a good correlation with the reference value (Ji et al., 1992). Additionally, Chi^2 test was used to evaluate the quality of fit between the experimental data and individual binding models. Typically, good fittings derive Chi^2 values less than 10. In our case, Chi^2 value was 9.71, which indicated that the BSA-modified chip was effective not only in binding the target proteins but also in suppressing the NSB of proteins.

Control experiment

CM5, the commercial chip, was employed to do the control experiment. This is a 100 nm think carboxymethyl-dextran matrix, which provides a relatively inert hydrophilic environment suitable for most biomolecular interactions and allows the use of a broad range of well-defined chemistries for covalently coupling of bimolecular

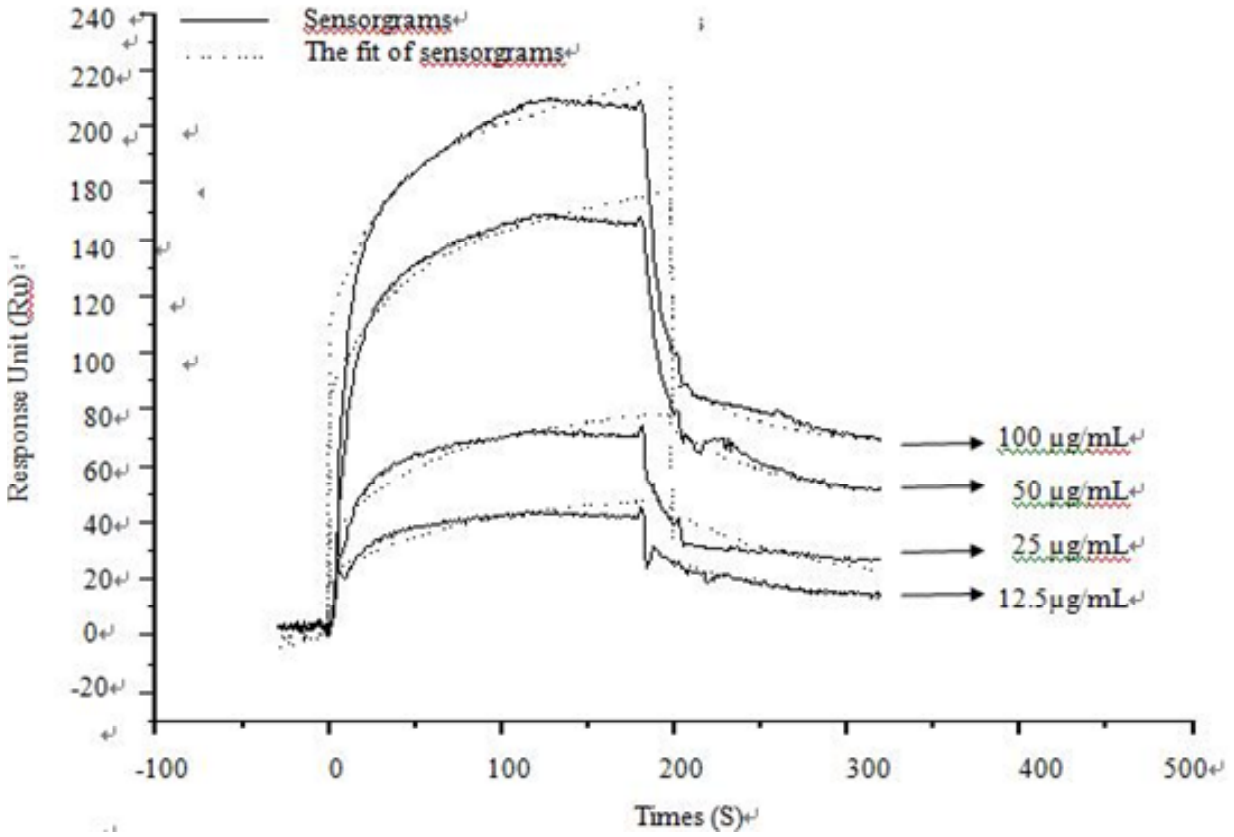

Figure 4. Solid lines showed the in-line signal changes of GSH/GST binding interaction with the subtractions of artifacts. Dot lines showed the fit of sensorgrams generated with BIAevaluation software.

to the surface. Meanwhile, dextran is also relatively flexible and so allows the "surface-attached" molecules to move with relative "freedom" in the matrix. CM5 chip was modified in accordance with the standard protocol (described in control experiment). First, only the experimental channel was active with NHS/EDC and GSH, then 50 μg/moL target protein was injected from active surface (Fc2) to inactive surface (Fc1). Unfortunately, a false signal covered up the real results. Therefore, the active/blocked CM5, that is the matrix modified with NHS/EDC, subsequently with mercaptoethanol, was designed as the reference surface. The utilization of mercaptoethanol is mainly to not only block the active function group from reference surface but also increase the hydrophilic surface. In this method, 4×10^9 target proteins per mm^2 were captured for experimental channel and 3.81×10^9 for reference channel (showed in Figure 5). The results were a slight improvement on the previous one but still not good. In contrast, the BSA-modified chip (94% difference) is more efficient to resist the background. The main reason why the GST binding to CM5 chip is three times higher than that of BSA chip is the electrostatic attraction between negative charges on the carboxymethyl dextran of CM5 sensor chip and positive charges on the GST. In fact, the low carboxyl Biacore CM4 sensor chip (60% net negative surface charge reduction) had been employed in our experiment. But the results were not ideal.

DISCUSSION

Recently, surface plasmon resonance (SPR) becomes more and more popular without the need of the label technology. However, sometimes, a number of experimental artifacts complicate the final biosensor analysis. The utilization of a reference surface can dramatically improve the quality of the binding data by correcting artifacts. In the design of the reference surface, a ubiquitous problem is to reduce non-specific binding effectively. Although nonspecific protein adsorption is complex and not well understand. It can be discussed in terms of two limiting mechanisms: adsorption by charge-charge interaction and adsorption by hydrophobic interaction (a combination of these two effects may, of course, occur) (Sigal et al., 1998; Chapman et al., 2000). In our early work, mixed SAM of alkanethiolates terminated hydroxy and amine functional group was employed for studies of biomolecular recognition. The chips were prepared by immersing into a series of solutions of varying concentrations of $HS(CH_2)_{11}NH_2$ and $HS(CH_2)_{11}OH$ with the total thiol concentration at 1 mM. The utilization of OH-terminated silane monolayer was not only to resist

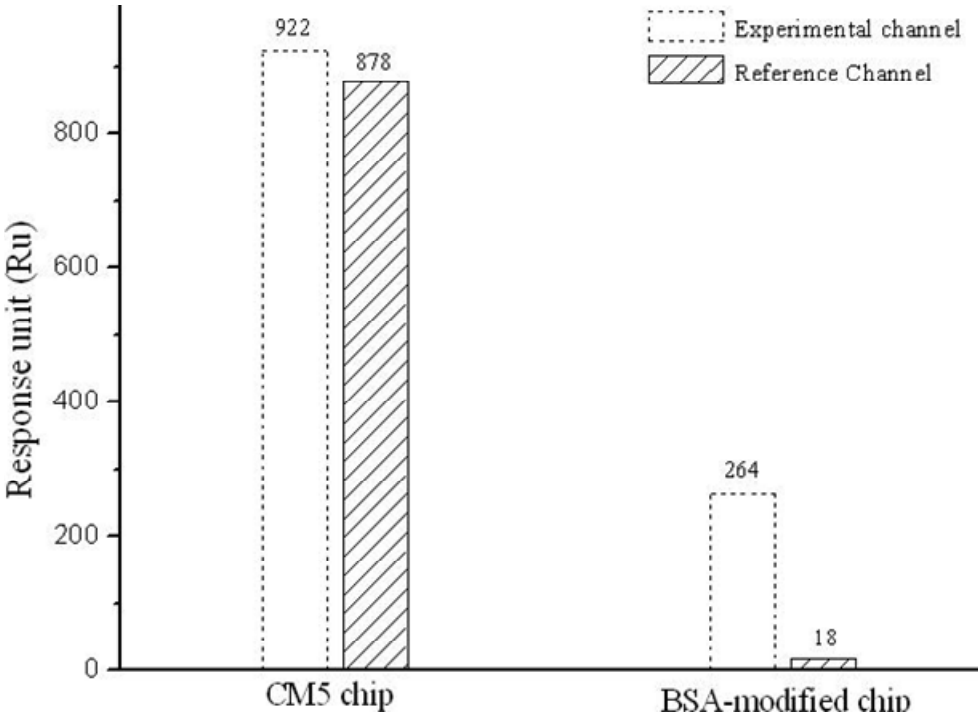

Figure 5. Solid lines showed the in-line signal changes of GSH/GST binding interaction with CM5 chip and BSA-modified chip for the reference channel, dot lines for experimental channel.

nonspecific adsorption of bimolecular but also to control the average surface density of ligands used for bio-specifics binding. Then, the sensitivity of NSB to the chip surface was studied with the different percentage OH and NH_2 concentration (1:1, 1:3, 1:5, 1:10, 1:15, 1:20). According to our previous results, we found that the signal recognition on the interaction of GSH/GST was getting worse while the minimal NSB appeared with the incense of OH groups. The best results were shown at 1:10. Unfortunately, it is unreliable due to the failed regeneration of the GSH-SAM matrix and the poor reference surface. At this case, we have to modify the reference channel with a non-interacting protein or with the activated/blocked surface, respectively. But two methods are failure. Thus BSA was used to passivate surface in order to prevent non-specific protein adsorption.

In the beginning of the work, BSA was introduced onto biochip surface only by the physical absorption. Because SPR response unit was seriously interfered from the desorbed BSA in high ionic strength solution, BSA had to be covalently bond with the chip finally. The conjugation of BSA was employed as a blocking motif as well as an amine group provider (Hirayama et al., 1990). There is somewhat spacing among amines because 59 lysines of BSA were distributed among 583 amino acids. It is similar to the conventional matrices (Mixed self-assembled mono layers) where the average surface density of functional groups can be adjusted by co-deposition of inert and active adsorbates. Both cases suffer from the random distribution of amines over matrices. At this case, the suitable protein binding site would reduce the steric effects during adsorption phenomena involving macromolecules (Lahiri et al., 1999; Chen, 2006).

Next, the BSA-bounded chip was used to study the kinetic behavior of GSH/GST and demonstrated its superior performance: the simply modification art, the low non-specific binding and the effective regeneration condition. All of those are crucial factors to successfully obtain the effective data. In addition, one more point worth mentioning is that many of the artifacts associated with binding data (which included nonspecific binding, bulk refractive index change, drift and jumps in signal) could be substracted successfully and matrix effect could be also ignored due to the slight difference from the active BSA surface conjugated with GSH (Fc2) to the unactive BSA surface (Fc1).

Another point I have to mention is that the low non-specific binding (aground 7.45%) was also demonstrated with the reference surface treated with BSA, GMBS and mercaptoethanol in succession (the activated/blocked BSA surface), which confirms the specificity of GSH/GST binding. However, due to an offensive odor of the blocking agent (mercaptoethanol) and a slight difference between the reference surface treated only with BSA and with BSA, GMBS and mercaptoethanol in succession, we still used the former modification art.

Finally, CM5, the most widely used chip in the study of ligand/protein or protein/protein interaction, was designed as the control experimental sample to prove further the validity of the BSA-bounded chip. Compared with the

unideal data resulted from the CM5, the BSA matrix show its own advantages and easily circumvents the shortcoming of the CM5 chip or the trouble of looking for the non- interacting protein.

Up to now, each protocol has its characteristic advantages and disadvantages; an optimized protocol has not yet been established. The utilization of BSA-bounded biochip in the investigation of kinetic constant maybe offer some unique advantages to circumvent the difficulties of other chips because the BSA surface can be directly regarded as a suitable reference surface to substract many of the artifacts associated with binding data (which included nonspecific binding, bulk refractive index change, drift and jumps in signal) without any modification.

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