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Development of Surface Plasmon Resonance Immunosensor through Metal Ion Affinity and Mixed Self-Assembled Monolayer

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An immunosensor based on surface plasmon resonance (SPR) with enhanced performance was developed through a mixed self-assembled monolayer. A mixture of 16-mercaptohexadecanic acid (16-MHA) and 1-undecanethiol with various molar ratios was self-assembled on gold (Au) surface and the carboxylic acid groups of 16-MHA were then coordinated to Zn ions by exposing the substrate to an ethanolic solution of $Zn(NO_3)_2 \cdot 6H_2O$. The antibody was immobilized on the SPR surface by exposing the functionalized substrate to the desired solution of antibody in phosphate-buffered saline (PBS) molecules. The film formation in series was confirmed by SPR and atomic force microscopy (AFM). The functionalized surface was applied to develop an SPR immunosensor for detecting human serum albumin (HSA) and the estimated detection limit (DL) was 4.27 nM. The limit value concentration can be well measured between ill and healthy conditions.

Keywords: Mixed self-assembled monolayer, surface plasmon resonance, immunosensor, metal ion, human serum albumin

Recently, the surface plasmon resonance (SPR) immunosensor is in the limelight of the academic field of immunoassays [13, 21]. There are three advantages: direct detection of analytes with high sensitivity, a short detection time, and simplicity [5, 19, 23]. Clearly, SPR is a powerful tool for its high resolution to identify chemical changes on metal thin films [9]. In the same principle, SPR can also be used in biomolecular binding [17], DNA hybridization [14], antibody-antigen recognition [2], epitope mapping [7], cell-based measurement [16], and protein nanoarrays [20], which were mainly feasible by fluorescence in the past [18]. However, the enhancement of its sensitivity is required to detect biological materials, as the concentrations of analytes in a biological system are extremely low.

The sensitivity of a SPR immunosensor can be increased by control of the orientation and 2-dimensional (2D) configuration of antibodies immobilized on the SPR sensor surface. When antibodies are immobilized on a solid surface, their activity is usually less than that of dispersed antibodies [11]. The main reason for activity reduction is due to the random oriented array of the antibody molecules and steric hindrance by antigen size in the binding characteristic between antibody and antigen on the solid surface. Therefore, the development of a immobilization method for antibodies is strongly required to make a highly oriented layer and minimize steric hindrance by antigen size in antibody-antigen binding characteristic.

Recently, several methods including physical and chemical adsorption have been proposed for preparing an oriented antibody molecular layer and/or minimizing the activity reduction of antibody layer on solid matrix surfaces. However, these approaches have depended on antibody binding proteins, genetic engineering technologies that produce binding tags for directed surface attachment, the use of covalent linker, or a combination thereof [4, 6, 12]. Although these techniques have been widely used, they have some problems such as expensive cost, extra labor, and complexity to inactivation due to denaturation of the antibody molecule, *etc.*

Here, we present an effective method of biomolecules immobilization for making an SPR immunosensor with

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enhanced performance. The metal ions could be utilized to immobilize unmodified antibodies in a way that endows the orientation of antibody molecules and preserves their biorecognition properties on the SPR sensor surface [26, 27]. This approach is related to ones used for immobilizing antibodies with metal ions on chromatographic supports. For minimizing the steric hindrance by antigen size in the binding characteristic between antibody and antigen, the 2D configuration of immobilized antibody molecules on the solid surface was controlled by molar ratio variation between 16-mercaptohexadecanic acid (16-MHA) and 1-undecanethiol. The film formation process was confirmed by SPR and atomic force microscopy (AFM). The study of sensitivity enhancement of the SPR immunosensor by simultaneously controlling the 2D configuration and orientation of immobilized antibody molecules of the sensor surface has not been reported yet.

The functionalized surface was applied to develop an SPR immunosensor for detecting human serum albumin (HSA). HSA is the most abundant plasma protein in the circulatory system, synthesized by the hepatocytes in the liver and translated as a preprosequence, targeting the protein to the secretary pathway [8]. HSA concentration can be a significant indicator for the early diagnosis of renal disease in diabetic patients.

Materials and Methods

Materials

The human serum albumin (HSA), 16-mercaptohexadecanoic acid (16-MHA, $HS(CH_2)_{15}COOH$), bovine serum albumin (BSA), 1-undecanethiol ($HS(CH_2)_{10}CH_3$), $Zn(NO_3)_2{\cdot}6H_2O$, phosphate-buffered saline (PBS, pH 7.4), and phosphate-buffered saline with Tween 20 (pH 7.4) were purchased from Sigma-Aldrich. Monoclonal antibody against HSA that does not have cross-reactivity with BSA was also purchased from Sigma-Aldrich. Cover glasses (BK7, Superior, Germany) were fabricated with a thickness of 2 nm chromium and 43 nm gold surface by DC (direct current) magnetron sputtering. Then, this gold substrate was cleaned by RF (radio frequency) plasma cleaning. H_2O was prepared with a water purification system (RO drain, Human Power I$^+$).

SPR Spectroscopy

The SPR measurements were performed through using SPR spectroscopy (Multiskop, Optrel GmbH, Germany) [10, 13]. A He-Ne laser with a wavelength of 632.8 nm was used as the light source. A prepared gold substrate was stuck to a BK7 prism with index matching oil (n=1.5168) and fixed on the holder. The resolution of the angle measurement in the goniometer was 0.001°. The detailed configuration is shown in Fig. 1A.

Immobilization of Anti-HSA Antibodies

To construct self-assembled monolayers (SAMs), prepared gold substrate was immersed in 16-MHA solution (20 mM in acetonitrile) for 12 h. After rinsing several times with absolute ethanol and deionized water, the carboxylic acid groups of 16-MHA were coordinated to Zn ions by exposing the substrate to an ethanolic solution of $Zn(NO_3)_2{\cdot}6H_2O$ (5 mM) for 8 h, followed by rinsing with absolute ethanol and deionized water, and the substrate was dried under a stream of N_2 gas to remove any residual materials. This functionalized substrate was exposed to a solution of 100 μg/ml anti-HSA (Sigma-Aldrich) in PBS buffer for 12 h at room temperature (Fig. 1B) [20, 21]. To confirm the effect of increasing concentration on anti-HSA, solutions of anti-HSA antibody were prepared in PBS with different concentrations such as 10 μg/ml, 20 μg/ml, 30 μg/ml, 50 μg/ml, and 80 μg/ml. Then, functionalized substrates were exposed to each solution of antibodies. After 12 h, the substrates were rinsed and dried.

Topological Analysis by Atomic Force Microscopy

The surface topographies of the prepared bare gold and antibody layer were obtained by atomic force microscopy (AFM; Auto Probe CP; Park Scientific Instruments, U.S.A.) operated in contact mode at room temperature under air conditioning. Images were acquired at a scan rate of 1.5 Hz with a silicon cantilever (Ultralever 06B; Park Scientific Instruments, U.S.A.).

Immunosensor with Mixed Self-Assembly Monolayer

Six different mixed SAMs were prepared as follows (molar ratios of 16-MHA and 10 undecanethiol are shown): SAM0 (without 1-undecanethiol, negative control), SAM1 (20:1), SAM2 (10:1), SAM3 (1:1), SAM4 (1:10), SAM5 (1:20), and SAM6 (1:30). Monolayers of self-assembly with mixed SAMs were made by using the above method. After binding Zn ions, anti-HSA antibody (300 μl of 30 μg/ml) was immobilized for 12 h. Then, the substrates were washed with PBS several times and cleaned with deionized water. Finally, the antibody surfaces were dried by N_2 pure gas. BSA solution (0.5%) in PBS was prepared as a classical blocking agent in solid-phase immunoassays. The substrates were immersed in prepared BSA solution for 1 h This procedure was needed for blocking the binding of unrelated proteins. After washing with PBS, the substrates were immersed in PBS with Tween-20 for 15 min, and cleaned with PBS and deionized water. Then, the substrates were dried with a stream of N_2 gas. A solution of HSA in PBS was made with constant concentration, 20 μg/ml. Immobilized antibody substrates were soaked in a 300-μl solution of HSA for 3 h. Then, the substrates were rinsed with PBS and deionized water and dried with pure N_2 gas. Finally, with an optimum 1:20 molar ratio self-assembled layer, the calibration curve of HSA detection by SPR sensor was completed.

Results and Discussion

Immobilization of Antibody Molecules on SPR Sensor Surface

Recently, it has been reported that zinc ions could be utilized to immobilize unmodified antibodies in a way that endows the orientation of antibody molecules and preserves their biorecognition properties on the solid surface [26, 27]. This approach is related to ones used for immobilizing antibodies with metal ions on chromatographic supports. They demonstrated how the Zn ions can be used

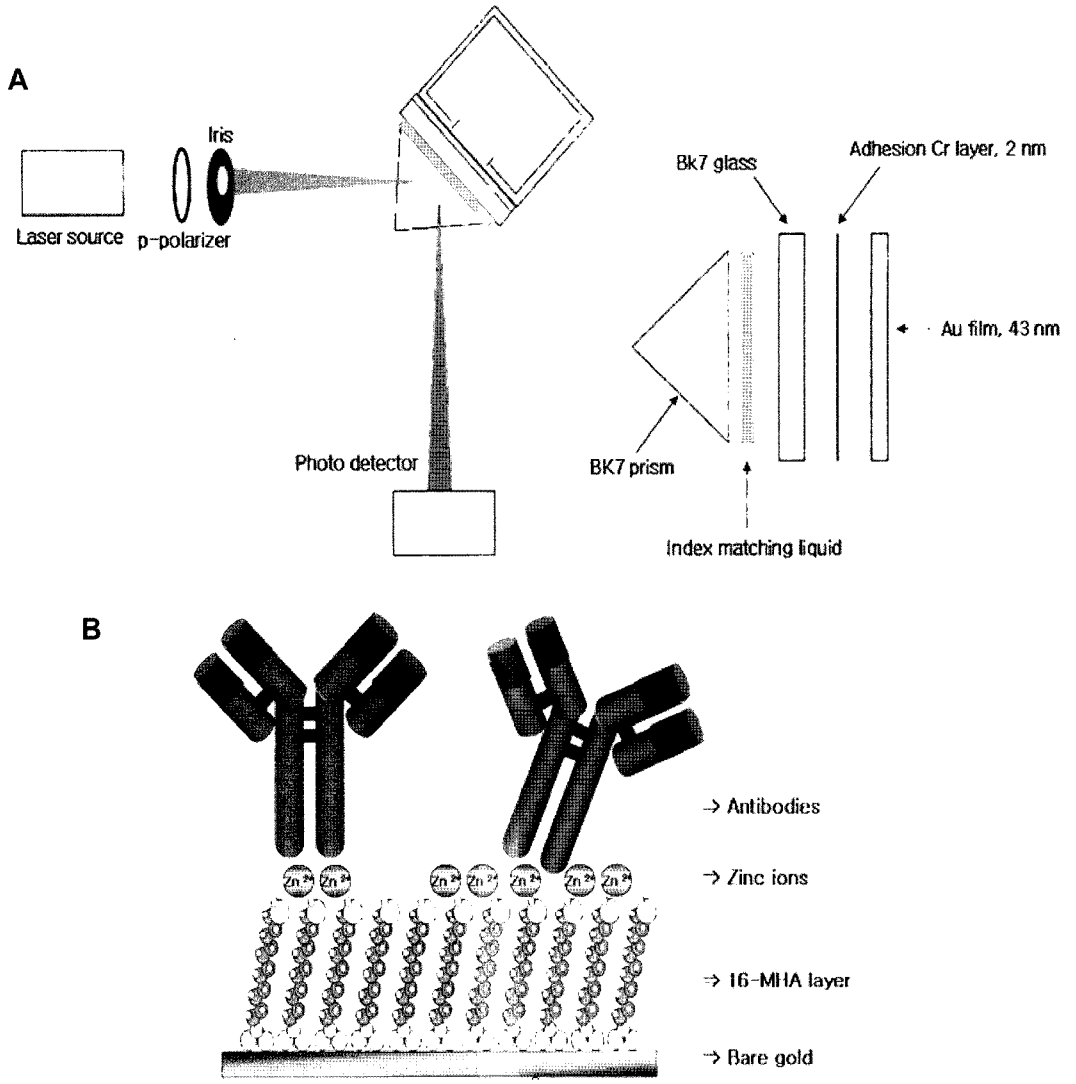

Fig. 1. **A.** Setup of SPR spectroscopy. **B.** Schematic diagram of antibody immobilization.

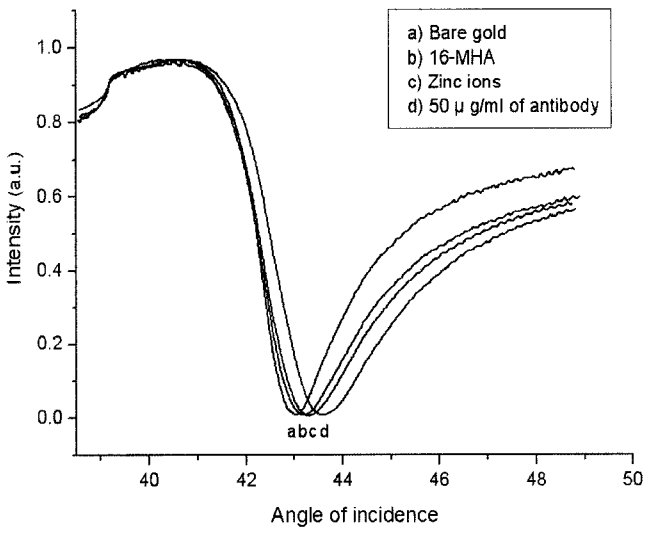

Fig. 2. Shifts of the SPR curve by adsorbing 16-MHA, Zn ions, and binding of antibody onto Au substrate in series (Lines: a, bare gold; b, 16-MHA; c, Zn ions; d, antibody).

as a versatile linker to immobilize antibodies on a solid surface in an active state.

The changes of SPR curves by adsorbing 16-MHA, the coordination of the zinc ions to the carboxyl terminal group of 16-MHA, and the binding of anti-HSA to the zinc ions by charge interaction in series on Au substrate are shown in Fig. 2. As a result, the SPR angle was shifted appropriately: 43.002°±0.002 on bare Au substrate, 43.182°± 0.002 on the 16-MHA layer, 43.210°±0.006 on the zinc ions layer and 43.536°±0.005 on the antibody layer. In principle, a surface plasmon is a bound electromagnetic wave propagating at the metal-dielectric interface. The external laser field drives the free electron gas of metal in a distinct mode. The spatial charge distribution creates an electric field, which is localized at the metal-dielectric interface. Hence, the plasmon resonance is extremely sensitive to the interfacial architecture. An adsorption process leads to a shift in the plasmon resonance and allows monitoring of the mass coverage at the surface with a high accuracy

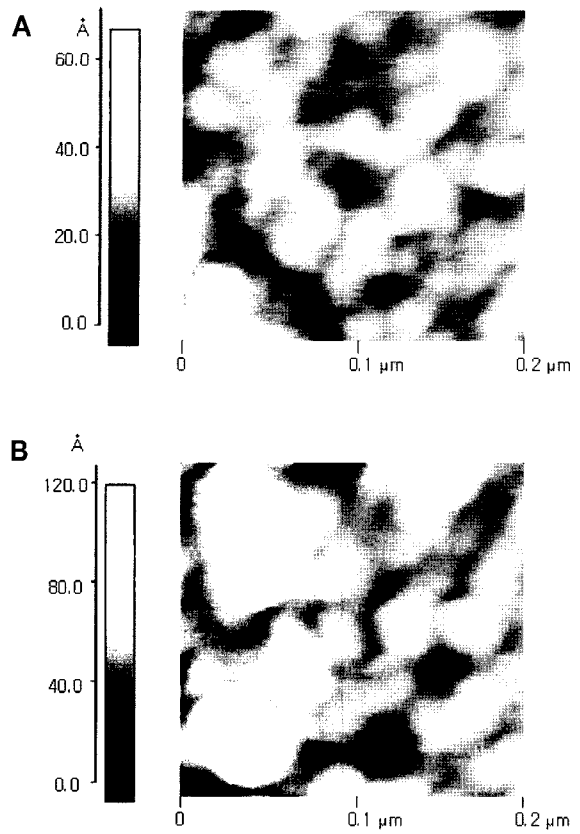

Fig. 3. AFM topography images.
A. Bare gold. **B**. Antibody layer (scan size 0.2 μm×0.2 μm).

[24]. Therefore, the shift of SPR angle verified that a thin layer of 16-MHA on the Au surface was formed, zinc ions were coordinated to the carboxylic terminal group of 16-MHA, and antibody molecules were well bound with the charged surface on the Au substrate.

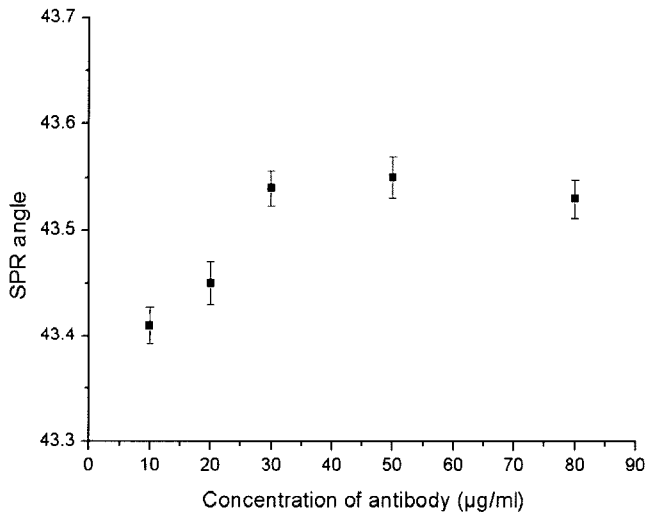

Fig. 4. The shifts of SPR angle by the adsorbing antibody as a function of various concentrations.
The size of the error bar shows 95% reliability.

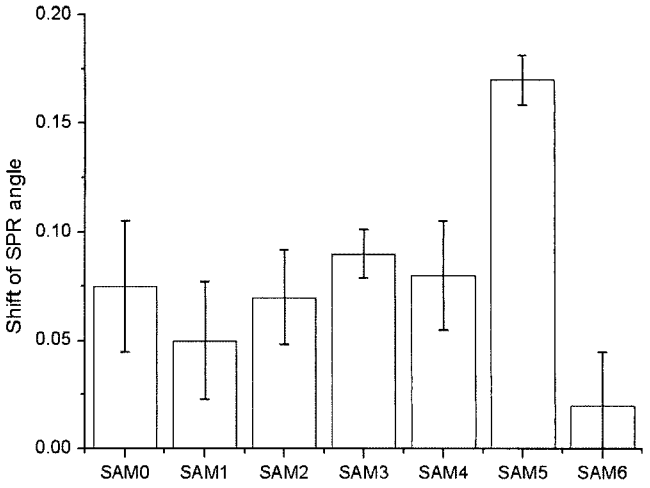

Fig. 5. The shifts of SPR angle by binding between antibody and antigen, where antibody layers were fabricated on the solid surface functionalized with Zn ion and various molar ratios of 16-MHA and 1-undecanethiol.
SAM0 (only 16-MHA), SAM1 (20:1 molar ratio of 16-MHA: 1-undecanethiol), SAM2 (10:1 molar ratio of 16-MHA: 1-undecanethiol), SAM3 (1:1 molar ratio of 16-MHA: 1-undecanethiol) SAM4 (1:10 molar ratio of 16-MHA: 1-undecanethiol), SAM5 (1:20 molar ratio of 16-MHA: 1-undecanethiol), and SAM6 (1:30 molar ratio of 16-MHA: 1-undecanethiol). The size of the error bar shows 95% reliability.

AFM images of bare Au and antibody layer formed on a Au surface, which was functionalized with 16-MHA and zinc ions, are shown in Fig. 3. To get more visible images, antibody substrate was not washed with detergents (usually Tween-20). It could be observed that the aggregated antibody protein clusters formed with a globular shape in a solid-like state with keeping its random cloud-like structure as in bulk solution. The size of these was different from that of bare Au. The height of the surface was changed from 60 Å (6 nm) to 120 Å (12 nm). From the above results, it could be concluded that an anti-HSA layer was fabricated on the Au substrate that was functionalized with 16-MHA and zinc ions.

As the concentration of antibody increased, the shift value of SPR angle was larger and the antibody surface loading was saturated at the concentration of 30 μg/ml (Fig. 4). From this result, the concentration of anti-HSA used in this study was determined to 30 μg/ml.

2D Configuration Control of Immobilized Anti-HSA

Recently, SAMs resulting from the co-adsorption of two different thiols (*i.e.*, mixed SAMs) have been shown to promote protein adsorption, as a result of multiple chemical functionalities on the surfaces, and to decrease steric hindrance around the functional tails [15]. In fact the stability and the molecular recognition properties of various proteins, once immobilized on mixed SAMs, were significantly improved as compared with pure SAMs layers. The reason why we used -CH_3 terminated thiol is that co-

adsorption of -COOH terminated thiol with hydrophobic thiols kept the carboxyl tail group in its acidic form [3].

In this research, the antibody-antigen binding efficiency on several types of antibody layers was investigated by monitoring the changes of SPR angle induced by the reaction between antibody and antigen (Fig. 5), where antibody layers were fabricated on the solid surface functionalized with Zn ion and various molar ratios of 16-MHA and 1-undecanethiol: SAM0 (only 16-MHA), SAM1 (20:1 molar ratio of 16-MHA: 1-undecanethiol), SAM2 (10:1 molar ratio of 16-MHA: 1-undecanethiol), SAM3 (1:1 molar ratio of 16-MHA: 1-undecanethiol) SAM4 (1:10 molar ratio of 16-MHA: 1-undecanethiol), SAM5 (1:20 molar ratio of 16-MHA: 1-undecanethiol), and SAM6 (1:30 molar ratio of 16-MHA: 1-undecanethiol).

The optimal molar ratio of 16-MHA to 1-undecanethiol for the formation of anti-HSA and HSA complex was 1 to 20. Ideally, binding the antibody in exposing the paratope is favored by dense packing of antibody molecules on the SPR surface. Dense packing by itself, however, is not sufficient for optimal antibody-antigen complex formation. Antigens need lateral access to the antibody paratope for unimpaired binding, and a larger antigen needs more space between antibodies to access the paratope than a smaller antigen. Thus, the effect of the steric hindrance by antigen size in the binding characteristics between antibody and antigen must be minimized for developing an SPR immunosensor with high efficiency. As a result, a 1 to 20 ratio (16-MHA to 1-undecanethiol) was the optimal condition to get maximum immunoresponse for HSA in the SPR system.

Surface Plasmon Resonance Immunosensing for Detection of HSA

As proof-of-concept for our novel immobilization scheme of antibody on the SPR surface, we evaluated the immunoresponse between anti-HSA and various concentrations of HSA, which is a biomarker of renal disease in diabetic patients. The signal relationship with respect to the HSA concentration is represented in Fig. 6. As shown in Fig. 6, the shift in SPR angle was increased in proportion to the concentration of HSA, and all target concentrations over the 3 nM to 300 nM concentration range could be easily differentiated from the negative control. The assay exhibited a linear relationship between the target concentration (notice that the x-axis is a log scale) and the SPR angle shift over a concentration range of two orders of magnitude. The estimated detection limit (DL) is 4.27 nM (9.47 μg/ml) after calculating with the standard deviation of the response and the slope. The detection limit of HSA from only the SPR detection technique in this study is much more sensitive than that of other SPR sensors that was reported in previous papers [22, 25].

Importantly, our SPR immunosensing system with the detection range of 3 nM and 300 nM can be efficiently applied to early diagnosis of diabetic patients, because the HSA concentration of a healthy person is approximately 20 μg/ml, whereas it can be up to 200 μg/ml in persons suffering from very bad renal disease [1]. From these results, it could be concluded that an immunosensor based on SPR with enhanced performance was developed through the mixed self-assembly monolayer for the detection of HSA. The fabrication technique of the SPR immunosensor used in this study for the detection of HSA could be applied to construct other immnosensors or protein chips with high efficiency.

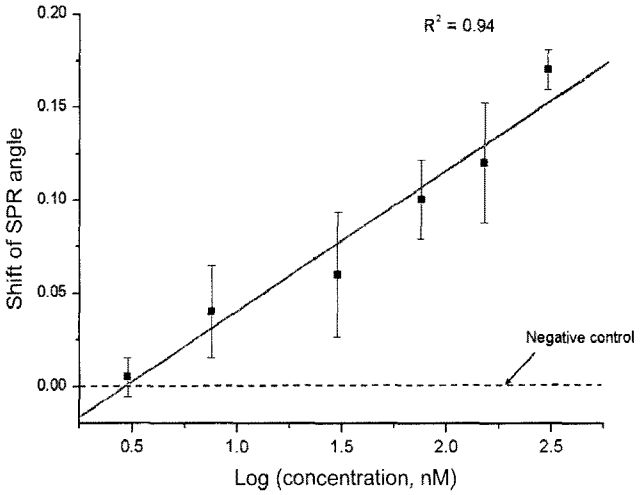

Fig. 6. Response of the SPR immunosensor as a function of HSA concentration.

In conclusion, an immunosensor based on SPR with enhanced performance was developed through the mixed self-assembly monolayer for the detection of HSA. The mixture of 16-MHA and 1-undecanethiol with various molar ratios was self-assembled on Au surface, and the carboxylic acid groups of 16-MHA were then coordinated to Zn ions. The antibody was immobilized on the SPR surface by charge interaction between the antibody molecule and Zn ions. The optimal molar ratio of 16-MHA to 1-undecanethiol for the formation of anti-HSA and HSA complex was 1 to 20. The functionalized surface was applied to develop an SPR immunosensor for detecting HSA and the estimated detection limit (DL) is 4.27 nM. The limit value concentration can be well measured between ill and healthy conditions. The fabrication technique of the SPR immunosensor used in this study could be applied to construct other immnosensors or protein chips with high efficiency.

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