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An Optical Biosensor for Monitoring Antigen Recognition Based on Surface Plasmon Resonance Using Avidin-Biotin System

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Abstract: A novel optical biosensor based on simultaneous multiwave length detection surface plasmon resonance (SPR) has been developed for immunosensing. The sensor is designed on the basis of fixing angle of incidence and measuring the reflected intensities of light in the wavelength range of 400–800 nm. The SPR spectra are shown in terms of reflected light intensity verus wavelength of incident light. The intensity of the reflected light is the minimum at the resonant wavelength. The biorecognition surface, formed on a chemically modified gold layer, consists of avidin that is specifically bound with biotin. These sensing membranes were self-assembled on gold layer. The processes of sensing monolayer formation were studied in real time through observing the change of resonant wavelength. The modified surface was used as a model immunosensor and to detect successfully the human factor B (Bf). The Bf was determined in the concentration range of 0.5~100 μg/mL. Under optimum experimental conditions, the sensor has a good repeatability, reversibility and selectivity.

Keywords: Surface plasmon resonance, Biosensor, Avidin, Biotin

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Introduction

Surface plasmon resonance (SPR) sensor is a useful analytical tool for studying the biomolecular recognition at surfaces because it can be used to monitor interactions between proteins and immobilized ligands in real time [1]. The basis of the immunosensing is the specific ligand interaction between the antibody and antigen, and the technique can directly sensing the refractive index changes in the close vicinity of a thin metal film surface on which the specific ligand is immobilized. An evanescent field is generated as light propagate with total internal reflection at the interface between glass and metal film, inducing the generation of surface plasmon from the free electrons in the metal film. The evanescent field from the glass-metal interface penetrates into the metal film and propagates with exponentially decreased amplitudes. Surface plasmon, which oscillates and propagates along the upper surface of the metal film, absorbs some of the plane-polarized light energy from the evanescent field, reduces the total-internal-reflection light intensity [2]. At certain angle or wavelength of incident light, the wave vectors of the surface plasmon and the evanescent field will be equal to each other, resulting in a resonance. As a result, the reflection light energy will be dramatically reduced at the resonant angle or wavelength. A plot of reflected light intensity versus incidence angle or wavelength yield an angular or wavelength intensity profile that displays a sharp dip. The exact location of the dip minimum (resonant angle or resonant wavelength) can be determined by detecting the change of the angle or wavelength. The binding of molecules being studied on the lower metal surface causes a change in refractive index of the surface medium that, in turn, can cause a shift of resonant angle or wavelength and thus can be used for determination of the species being studied [3].

If the wavelength of incident light keeps the same, the reflective intensity is the least at certain angle when SPR happens. On the other hand, if the angle of the incident light is fixed, the reflective intensity will have a minimum at certain wavelength. Based on the latter theorem, various simultaneous multi-wavelength measurement SPR sensors were developed [4-16].

The sensing principle of most commercial SPR devices is based on fixing a discrete excitation wavelength and modulating the angle of incident light, therefore, the SPR reflected spectra were shown as a function of the angle of incident light. In practice, the mode of operation is to use a huge machinery-rotating shelf. It makes use of a coupling prism coated with a thin gold film and performs the sensing by varying the incidence angle with a goniometer [17]. The best angular accuracy of the goniometer is about 0.001^{o} which corresponds to a shift in optical wavelength of 0.6 nm [18,19]. The other is to make use of the diffuse effect of a spot light to measure the intensity of the refracted light with a detector matrix [20]. In this case, the angle to be measured is limited. With BIAcore instrument, which was commercialized by Pharmacia Biosensor AB (Uppsala, Sweden) of this technology in 1990, many sensitive biochemical sensors have been developed and applied to the investigation of biomacromolecular interactions [21-23], especially to the immunochemical sensing area based on antibody-antigen interaction [24-26] . However, the BIAcore instrument is quite expensive.

A fiber optical SPR sensor (FOSPR) based on a limited range of incident angle and modulating the excitation wavelength was reported by Jorgenson et al [5,6]. Since a multi-mode optical fiber is used as a sensing element, the sensor is compact. Similar FOSPR was developed by Quinn et al [14]. The system was suitable for the analysis of crude samples and provided a means of monitoring red blood

cell-ligand interactions in real time at high concentrations. A commercial SPR sensor, the BIACORE Probe, is also a good FOSPR biosensor.

High-sensitivity stark spectroscopy was also obtained by using for the analysis of macromolecular analytes contained in crude matrices SPR [15]. And a high-resolution multiwavelength SPR spectroscopy was developed for probing conformational and electronic changes in redox proteins [16].

The SPR wavelength of colloidal gold particles coated with a monoclonal antibody was shown to be red-shifted when the antibody interacted with its specific ligand to produce a change in the refractive index [8]. This property has been used to monitor in real time the association and dissociation kinetics of the interaction in solution.

The new sensor developed in this study is making use of a coupling prism [9-12]. However, instead of employing a fixed wavelength and modulated the incidence angle of light, the incident angle is fixed and the excitation wavelength modulated. A halogen tungsten lamp was used as the light source and a charge coupled device (CCD) detector was used for monitoring the whole wavelength range of 400-800 nm, simultaneously. With this setup, changes in the sensing of the membrane will give rise to a shift of resonant wavelength. Because a change in concentration or kind of analyte in the flow cell will lead to the change in refractive index of the layer of the sensing membrane. It has two obvious characteristics that distinguish it from other SPR sensors. First, there is no moving part in the sensor. Second, the sensor is measuring reflected intensities at all wavelengths in the range of 400-800 nm simultaneously. That is to say, it can monitor reaction in real time. The system has been used for the determination of some chemical species, such as dimethylamine, alcohol and glucose [9]. This paper presents an improved device that can be used as both a molecular recognition device for detecting related biomolecules and a tool for studying macromolecular interactions including measuring antibody-antigen, and ligand-receptors kinetic association constants [10-12]. The sensor was shown to be of simplicity, sensitivity, selectivity, rapid response and cost effectiveness.

In recent years, self-assembling techniques have gained increasing attention for processing antibody molecular organized on the solid surface. To assembly a self-assembling monolayer (SAM) on gold surface is relatively easy and the SAM is usually closely packed, well ordered and stable under ambient conditions. Thus, SAM is a good candidate for immobilizing biomolecules on gold or sensing membrane.

The strong and specific noncovalent interaction between biotin (vitamin H) and the egg-white glycoprotein avidin (MW 68 000) or the nonglycosylated protein streptavidin (MW 60 000) leads to the formation of a high-affinity complex (K_a is about 10^{-15} M) [27] which can easily visualized by enzymatic methods that are commercially available. These properties have been exploited for the detection or localization of proteins, carbohydrates and nucleic acids [28]. Once avidin is on the surface, it is simple to immobilize any biomolecules with a biotin label. Avidin is a tetramer protein that has four identical binding sites for biotin. The binding of biotin to avidin is almost irreversible. The complex is virtually unaffected by extreme pH, temperature, and organic solvents and other denaturing agents [29]. The tetravalency of avidin for biotin allows the construction of a "molecular sandwich" that allows the surface-bound avidin to be coupled to a biotinylated antibody that has the appropriate characteristics needed for the construction of the biosensor developed in this paper.

Experimental

Reagents and Materials

Dithiodiglycolig acid ($C_4H_6O_4S_2$, DDA) and avidin (from egg white, extrapure reagent) were obtained from Sigma. *N*-Ethyl-*N'*-(3-(dimethyl)aminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were obtain from Shanghai Lizhu Dongfeng Biotechnology Co. Biotinylated anti-rabbit IgG were obtain from Beijing Zhongshan Biotechnology Co. Rabbit anti-human factor B antiserum (rabbit IgG fraction to human Bf, titer 1:60), human factor B, human serum albumin were purchased from Shanghai Biology Product Research Institute, PRC. All other chemicals were of an analytical reagent grade. All solutions were prepared with ultrapure water (>18 $M\Omega \cdot cm^{-1}$) supplied by EASYpure RF compact ultrapure water system (Barrnstead Thermdyme Inc., USA).

0.01 mol/L phosphate-buffered saline solution (PBS, pH=7.4) were prepared containing 0.2 g KCl, 8.0 g NaCl, 0.24 g KH_2PO_4 and 1.44 g $NaHPO_4$ dissolved in 1000 mL ultrapure water. 0.3 mol/L citrate buffered saline solution (pH=2.7) were prepared by dissolving 21 g $C_6H_8O_7 \cdot H_2O$ and 11 g Na_2HPO_4 in 350 mL ultrapure water.

SPR set-up

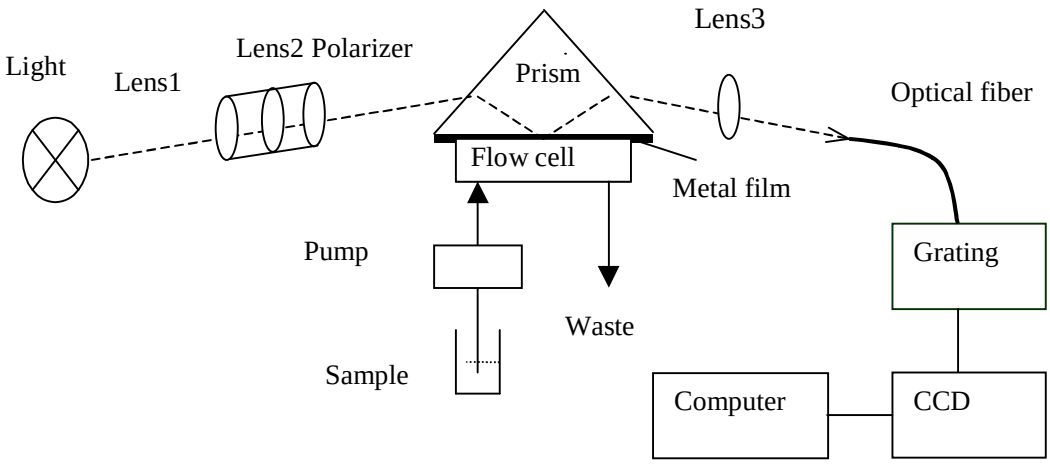

Figure 1. Block diagram of SPR sensor based on simultaneous multi-wavelength detection.

The schematic diagram of the SPR optical immunosensor is shown in Figure 1. The light source is a halogen tungsten lamp in conjunction with a constant voltage transformer. The light from this source passes through a polarizer and becomes TM polarized light. In order to make the light be parallel one, two lenses are employed. The focal lengths of lens 1 and lens 2 are 30 mm and the working distance from lens 1 to lens 2 is 60 mm. The exit light from the prism is guided into the optical fiber and then to the Fullwave Spectrophotometer (Ocean Optics, Inc. USA). The spectrophotometer was inserted into a computer, therefore, the apparatus is compact and small. The focal length of lens 3 is 75 mm. The detector is a 1024 element CCD linear-array detector and the wavelength range to be monitored simultaneously is 400-800 nm.

The incident angle is fixed at a suitable value to ensure the surface plasmon resonance phenomenon to occur. The reflected light intensity is the minimum at the resonant wavelength. A smaller increase in refractive index of the analyzed solution would cause a clear shift in SPR reflected spectra towards longer resonant wavelength. A small volume flow cell (0.18 mL) was used for reaction.

The prism was vacuum-deposited with a gold film of 50 nm. To deposit the gold film on the prism, the prism was mounted in an electron-beam evaporator system with the flux of evaporated metal perpendicular to the prism (K9 glass, n=1.567). The deposition process was monitored using a quartz crystal detector.

The gold SPR surface was cleaned by exposure to ethanol for 5 min, followed by drying with nitrogen.

Self-assembled monolayers

Molecular self-assembly in solution is used to form a sensing membrane on the gold substrate. An ethanolic solution of 3,3'-dithiodiglycolig acid (DDA) was injected into the flow cell with a syringe. The gold surface of the prism was exposed to 5 mmol/L DDA for 1 hour, followed by water rinsing. 0.25 mL of 100 mg/mL EDC solution and 0.25 mL of 100 mg/mL NHS solution were mixed, and then were injected into the flow cell. This solution was allowed to interact with the DDA for 1 hour. The surface was then rinsed with water and immersed in an aqueous 0.2 mg/mL avidin solution for at least 1 hour, after which the surface was rinsed with water again. A PBS solution containing 5% bovine serum albumin (BSA) was used to block the nonspecific binding sites. Then a 2 μg/mL biotinlated anti-rabbit IgG PBS solution was injected into the flow cell and kept to immerse the sensing membrane for 40 min. After the biotinlated anti-rabbit IgG monolayer was formed, 2 mL of 0.01 mol/L PBS (pH=7.4) was used to wash off the unbound antibody for several times.

The diluted rabbit anti-human factor B antiserum (1:10) was injected into the flow cell after the biotinlated anti-rabbit IgG monolayer formation. The unbound antibody was taken away by washing several times with PBS.

All procedures were performed at room temperature, 20±1 ^{o}C. Taking the SPR spectra at different time to monitor the process of the monolayer formation.

Determination of factor B. 0.01 mol/L PBS was used to diluted different concentrate of human factor B (BF). Then BF was injected into flow cell and let it stay there for 30 min. The determination of the concentration was also by taking the SPR spectra with the computer. All procedures were performed at room temperature, 20±1 ^{o}C.

Results and Discussion

Monolayer formation

The DDA monolayer stickes firmly on the gold surface with its disulfides bond. To observe the DDA assembling on gold substrate, a solution of DDA was injected into the flow cell. The changes of resonant wavelength were determined in real time. The shift of the resonant wavelength reaches about 99% of its total shift within 25 min in the 6.7 mmol/L DDA solution. Further increasing the assembling

time, the Δλ keeps almost constant. That means the self-assembly has completed and the monolayer formed. The disulfide bond of DDA is easy to form S-Au binding. Meanwhile –COOH group reacts with NHS. Therefore, DDA forms a good monolayer. The resonant wavelength shift for the formation of this monolayer is 3.24 nm. NHS reacted with DDA under the catalysis of EDC. Then it was rinsed with water for several times, until the spectrum is stable. The maximum shift of resonance wavelength is 2.89 nm.

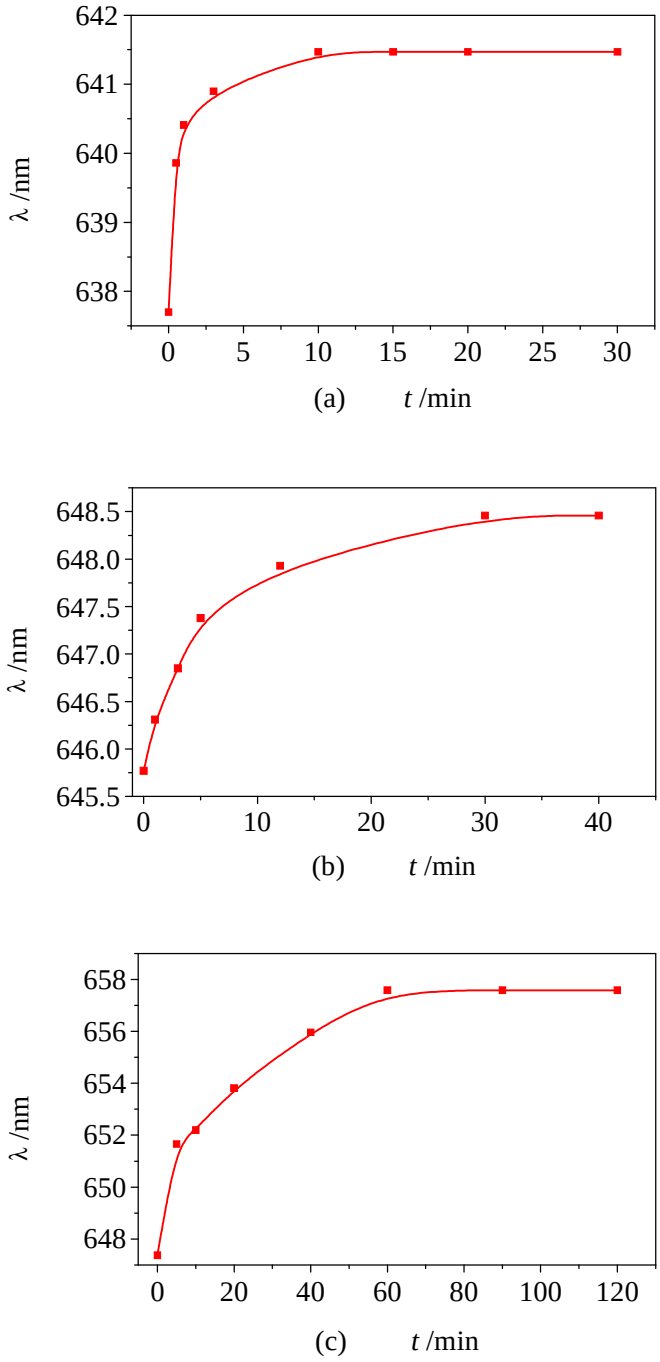

Figure 2. Resonant wavelength shift as a function of time for binding on the sensor surface. (a) avidin, Avidin concentration:0.2 mg/mL; (b) biotinylated anti-rabbit IgG, biotinylated anti-rabbit IgG titer: 1:20; (c) rabbit anti-human Bf antibody.

The $-NH_2$ group of avidin can easily exchange with the –NHS of the sensor surface. The adsorption curve of avidin (from 0.2 mg/mL aqueous solution) on the surface of sensor was shown in Figure 2(a). The maximum shift of the resonant wavelength was 4.5 nm and becoming stable in 10 min. In order to make the avidin monolayer well ordered and stable, the sensor surface was immersed in avidin solution for 60 min, then it was rinsed with pure water for several times. The shift of the resonant was then kept constant. The avidin monolayer should not be dried, as avidin was found to be denatured on drying [30].

The surface was rinsed with water again. The large excess of 5% BSA was used to block the nonspecific binding sites on the sensor surface. Then a 2 μg/mL biotinylated anti-rabbit IgG PBS solution was injected in the flowing cell and kept to immerse the sensing membrane for 40 min. Figure 2(b) showed the resonant wavelength shift as a function of time for adsorption of biotinylated anti-rabbit IgG on the surface of avidin. SPR spectra of biotinylated anti-rabbit IgG assembling on the surface of avidin monolayer were shown in Figure 3. Immobilization is confirmed by the increase of resonant wavelength shift, $\Delta\lambda$=2.69 nm, and is completed in 30 min. After the biotinlated anti-rabbit IgG monolayer formation, the sensor surface was rinsed with PBS buffer for several times. The resonant wavelength was kept stable. The value of adsorption constant (Ka) is calculated to be 1.15×10^6.

Bf is an important complement activator of complement 3 (C3), which plays a crucial role in the activation of the alternative pathway of C3 on the surface of biomaterials during extra-corporeal procedures. The content of Bf is related with diabetes, hypertension, and so on. In order to detect human Bf, a rabbit anti-human Bf antiserum was injected into the flow cell of the SPR sensor. The antibody assembling was monitored again in real time. The assembling conditions including antibody titer, pH value and reaction temperature were optimized. Figure 2(c) shows the time dependence of biotinylated anti-rabbit IgG monolayer assembled with rabbit anti-human B factor antibody at 20 ^oC. The assembling of the antibody (1:10) was carried out for 1 h to organize the processing antibody molecular on the solid surface and thus to stabilize the sensor membrane.

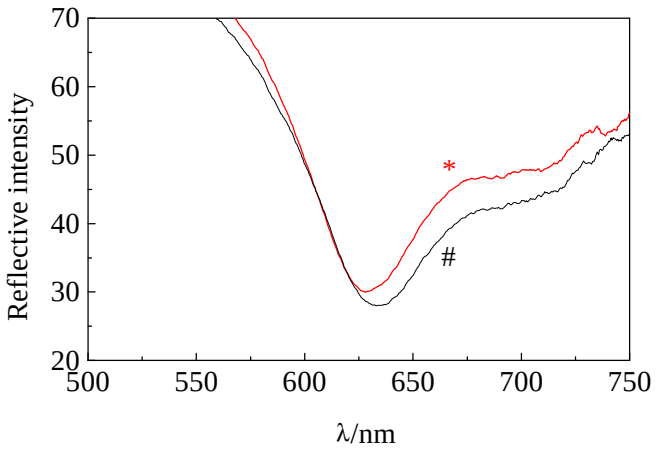

Figure 3. Resonant wavelength shift versus immobilization time of biotinylated anti-rabbit IgG onto an avidin-modified surface of SPR sensor: * Avidin, #. biotinylated anti-rabbit IgG.

Determination of Bf

A series of standard Bf solutions was prepared. The optimum determination conditions were shown to be in PBS buffer (pH 7.4) at 20 ^{o}C for 30 min. Room temperature (20 ^{o}C) was chosen for the antigen and antibody assembling. Figure 4 shows the relationship between Bf concentration and resonance wavelength shift. The Bf was determined in the concentration range of 0.5 ~100 μg/mL. Repeating the determination of 15 μg/mL Bf solution for 11 times, a relative standard deviation of 1.2% is obtained.

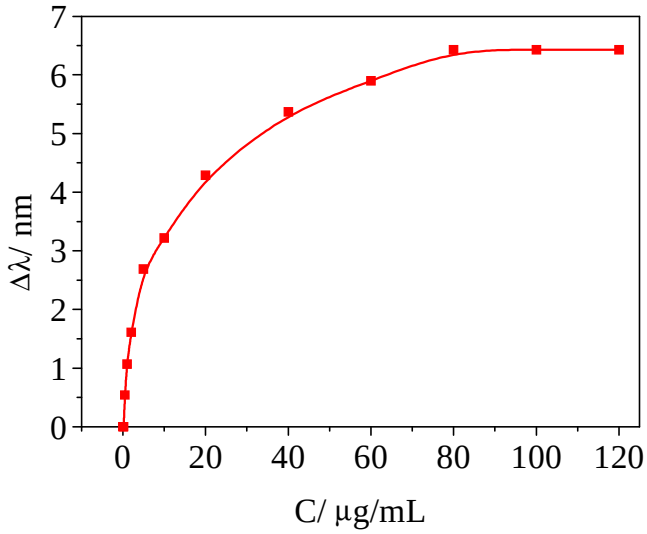

Figure 4. The relationship between concentration of Bf and resonance wavelength change.

Selectivity

Under the optimum conditions, 3.5 mg/mL human serum albumin and 5 μg/mL C_4 were then injected into the flow cell after the anti-human Bf antibody monolayer formed. It was found that these major components of human serum did not interfere the determination of Bf when Bf concentration is over 5 μg/mL. The immunosensor has good selectivity.

Regeneration

The bound antibody can be eluted from biotinylated anti-rabbit IgG with acidic solution. Several solutions were tried to elute the antigen-antibody complex. Figure 5 (a) shown the elution curve of the sensor membrane after being rinsed with 0.3 mol/L citrate buffer (pH=2.7). The sensor response decreases slowly for the first several times regeneration. The sensor can be used repeatedly about 50 times (Figure 5 (b)). The antigen-antibody complex is eluted from the avidin-biotin surface within 10 minutes. Be careful never to let the samples stay in the flow cell for long time because it will make the complex difficult to be rinsed and waste the sensor membrane.

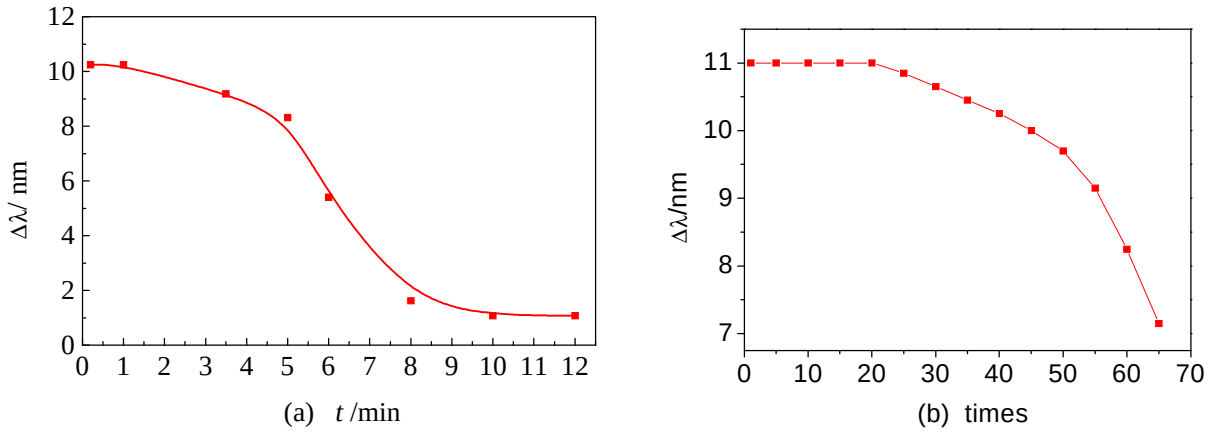

Figure 5. The regeneration of the sensor. (a) The elution curve of antibody-antigen composite from sensor membrane. (b) The resonance wavelength changes of the sensor after successive regeneration with 0.3 mol/L citrate buffer.

Conclusions

Simultaneous multiwavelength detection system based on surface plasmon resonance technique was developed. The sensor with avidin-immobilized biotinylated antibody can be successfully used for in situ detection of an antigen. The resonant wavelength shift is a function of concentration of the antigen in samples. The data presented provide a promising base for the development of immunosensors. In particular, the combination of a real-time monitoring of the SPR spectra and the specific interaction of an avidin-biotin system makes an attractive biosensor possible for rapid detection of various antigen, receptor, or DNA species.

Acknowledgments

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Sample Availability: Available from the author.