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A Label-Free Multisensing Immunosensor Based on Imaging Ellipsometry

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An immunosensor based on imaging ellipsometry and its potential applications was demonstrated in this paper. It has been proven a fast, reliable, and convenient method to quantify the thickness distribution of protein layers or detect protein concentration in solution. Combined with a protein chip, the immunosensor was able to detect multiple analytes simultaneously without any labeling. Preliminary results demonstrated how this immunosensor could be used to monitor several independent biospecific binding processes in real-time and in situ conditions.

Immunosensors have been the subject of increasing interest during the past decade because of their potential applications as an alternative immunoassay technique in many areas such as clinical diagnostics, environmental control, and biological research. As an optical technique, optical immunosensors are nondestructive and can be used in any environment that allows light access to samples. Some of optical immunosensors can be directly used to monitor the binding process of an antibody to its specific antigen without any labeling, such as surface plasmon resonance,[1] reflection interference spectroscopy,[2] and ellipsometry,[3−6] etc. The main advantage of label-free methods is that they avoid disturbances from conjugated markers or handling with radioactive materials, which allows their applications in a broad range of different biological systems.

As early as 1945, ellipsometry was used as an immunosensor to study antigen−antibody interaction.[7] Ellipsometry is a nondestructive, label-free optical method for determining film thickness with a resolution of 0.01 nm or better.[8] As an optical technique, it does not need an ambient vacuum and can be conveniently used in air or liquid. Fast ellipsometry methods, single wavelength or multiwavelength, have been adopted for monitoring film growth in situ, allowing precise control of film deposition processes.[8,9] Imaging ellipsometry is an enhancement of standard single-beam ellipsometry that combines the power of ellipsometry with microscopy.[4] A high spatial resolution in the order of micrometers (laterally) and subnanometers (vertically) can be achieved in bioaffinity-based sensing by the immunosensor based on imaging ellipsometry.[10] The enhanced spatial resolution of imaging ellipsometry potentially expands ellispometry into new areas of biological analysis. The field of view of the immunosensor is so large (several square centimeters) that it is easy to obtain the quantitative thickness distribution of a large area of biomolecular film. Another advantage is that it allows multisensing. An affinity biochip with a matrix of 900 targets has been detected with imaging ellipsometry.[5] In this paper, a patterned silicon wafer was used to fabricate a protein chip. The immunosensor, combining with the protein chip, was able not only to detect multiple analytes simultaneously but also to monitor multiple interaction processes in real-time and in situ conditions.

EXPERIMENTAL SECTION

Materials. Bovine serum albumin (BSA), goat anti-BSA serum, human serum albumin (HSA), goat anti-HSA serum, human immunoglobulin G (IgG), goat anti-IgG serum, human fibrinogen (Fib), and goat anti-Fib serum were obtained from Sigma. Water was obtained from a Millipore Milli-Q ion exchange apparatus. Chemicals used for the buffer preparation were all of analytical grade or better. Silicon wafers were purchased from the General Research Institute for Nonferrous Metals.

Substrates. The surface of the substrate for the ellipsometry experiments should be optically flat and reflecting. The polished silicon wafer used for the construction of electronic devices is a highly suitable substrate for ellipsometry experiments. Its surface energy, polarity, and surface chemistry can be easily controlled by silanization. Therefore, the polished silicon wafer used for the fabrication of electronic devices was chosen as substrate in this study.

Preparation of Probe and Detection. The probe was fabricated to detect a single sample for one time. The silicon wafers were cut into rectangles about 0.3 cm by 1.5 cm and made hydrophilic by immersing in solution (30% H_2O_2:98% H_2SO_4 = 1:3 (v/v)) for 30 min. The solution could not only remove contaminants of the silicon surface but also improved the number of silanol groups on the surface, thus making surface hydrophilic. The hydrophobic surface was prepared by silanization of the hydrophilic surface. After rinsing in distilled water and ethanol, the

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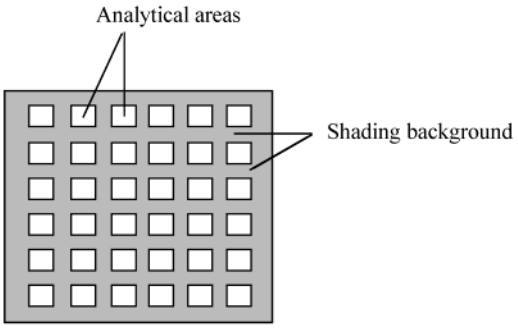

Figure 1. Sketch of the protein chip surface. The dimension of each analytical area is 0.75 × 0.75 mm^2.

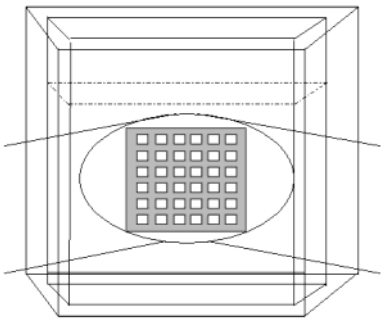

Figure 2. Sketch of the reaction cell with a protein chip illuminated by the probe light beam. The volume of the cell is 1 mL.

hydrophilic surfaces were incubated in a mixture of dichlordimethylsilane (20%, v/v) and trichlorethylene (80%) for 5 min, followed by rinsing in sequence with ethanol and trichlorethylene. Through reaction of dichlordimethylsilane with silanol surface groups, $Si(CH_3)_2$ was covalently immobilized to form a layer of densely packed methyl groups on a silicon dioxide layer and make the surface highly hydrophobic, with an advancing contact angle of ~75°.

The hydrophobic surface was incubated with BSA solution (1 mg/mL, in PBS buffer (10 mM phosphate, 0.1 M NaCl, pH 7.2) for 30 min at room temperature. The surfaces were rinsed with water to remove the nonadsorbed molecules and dried with a stream of nitrogen.

The BSA layers were incubated with anti-BSA solutions of different concentrations (0.1, 1, 3, 5, 10, 20, and 30 μg/mL, diluted with PBS buffer) for 30 min at room temperature. The anti-BSA in solution would interact with the immobilized BSA on the surface to form antibody−antigen complexes. The surface concentration in the area where the interaction took place became higher than before exposure to the solution. The change of the surface concentration was measured by imaging ellipsometry. The calibration curve was obtained by plotting the surface concentration versus the different concentrations of anti-BSA.

Preparation of Protein Chip and Detection. The preparation of the protein chip included the following steps. (1) Surface patterning of substrates: substrates for the protein chip were patterned by the standard photolithographic and wet chemical etching technique.[11] The surface of chips had many square-shaped analytical areas, each with a dimension of 0.75 × 0.75 mm^2, which were isolated with shading background (1−μm depth). The distance between both centers of neighboring areas is 1 mm. The format of the surfaces was shown in Figure 1. (2) Surface modification: the surfaces were made hydrophobic by silanization with dichlordimethylsilane by the method described in the preparation of the probe. (3) Immobilization of ligands: the solutions (1 mg/mL) of Fib, IgG, HSA, and BSA were dropped individually on analytical areas by pipet. After 30 min at room temperature, the chip was rinsed with water to remove all the nonadsorbed molecules and then dried under a stream of nitrogen. The thickness distribution of saturated protein layers on the protein chip fabricated by this method was homogeneous. The surface concentrations of these proteins were measured by the immunosensor based on imaging ellipsometry. (4) Detection: the protein chip was immersed in a mixture of three antiserums which were anti-Fib, anti-IgG, and anti-HSA; each protein concentration was 0.1 mg/mL. After 30 min at room temperature, the chip was rinsed with water and dried with nitrogen. The surface concentrations of analytical areas were measured with the immunosensor based on imaging ellipsometry.

Reaction Cell. A reaction cell was designed for real-time monitoring of the biomolecule interaction between the analytes in solution and the ligands on the silicon surface. The volume of the cell was 1 mL, and it had two optical windows for the probe light beam of imaging ellipsometry in and out. The normal of the two windows was parallel to the incident direction and the reflection direction of the probe light. The windows with optical quality were transparent for the probe light. The analyte solution could be poured into the cell and pumped out by a pump. The structure of the cell was shown in Figure 2.

In the real-time experiments, the cell was located on the sample supporter crossing the probe beam. The protein chip coated with multiareas of Fib, IgG, and HSA fabricated by the method described above was inserted into the cell and illuminated by the main beam of the probe light. A mixture of three antiserums, anti-Fib, anti-IgG, and anti-HSA, was poured into the cell; each protein concentration was 25 μg/mL. The antibodies in the solution reacted with their corresponding antigens on the surface according to the affinity of biomolecule interaction; thus, the surface concentrations of biomolecules in analytical areas increased. The growth of the surface concentrations was recorded with the immunosensor based on imaging ellipsometry in a series of images via time. The binding curves in real time were obtained by plotting the surface concentrations versus the reaction time.

Immunosensor. The experiments were carried out with an immunosensor based on imaging ellipsometry developed in our laboratory. Imaging ellipsometry was an enhancement of standard single-beam ellipsometry, which combined the power of ellipsometry with microscopy and worked in the off-null mode.[4] The imaging ellipsometry used in this study was an automated one, and ellipsometric conditions could be controlled by autoadjusting the polarizer and the analyzer; the angle of incidence was variable from 45 to 90° with a resolution of 0.05°; the magnification of image to object is modulated according to the dimension of the field of view, so that the lateral resolution of 3 μm could be reached; autofocusing was realized with the standard of the Laplacian algorithm. All the adjustments were carried out automatically with microstepping motors controlled by a computer with homemade software. With the same software, the digital images in gray scale format

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(8 bits, 0−255 Gycale) could be also automatically captured and processed. The light source was a xenon lamp, and a specific collimating system was used to provide an expanded parallel probe beam with a diameter of ∼25 mm. The beam passed through a polarizer and a compensator (a quarter wave plate) and finally onto the sample at an incident angle of 75°. An optical filter at 633-nm wavelength was placed in the incident optical passage to select a wavelength in order to increase the ellipsometric contrast of image. The reflection beam passed through an analyzer and an imaging lens with a spatial filter located at its focus plane, and then the ellipsometric image was focused onto the sensing area of the CCD camera. For a sample with lateral distribution of layer thickness (or surface concentration), null ellipsometry could not be carried out over the entire surface simultaneously due to the fact that different areas would yield different polarization changes. To overcome the problem, the optical components in the immunosensor were adjusted to fulfill the null conditions on a silicon wafer without adsorbed layers, and the off-null ellipsometric principle was used to measure the adsorption layer thickness (or surface concentration). Under this condition, the detected intensity "I" was related to the thickness (d) of the layer according to $I = kd^2$. As for the same protein and the same ellipsometric conditions, k is a constant and can be determined by the protein layer with known intensity in gray scale and its absolute thickness. In this paper, the absolute thickness of the protein layer was calibrated by conventional ellipsometry. The relationship between surface concentration and protein layer thickness was, surface concentration (μg/cm^2) ≈ Kd (nm), where $K \approx 0.12$[12].

Ellipsometer. The thickness of biomolecular layers was calibrated with a rotating analyzer-type ellipsometer (SE 400, Sentech) equipped with a He−Ne laser (λ = 632.8 nm). The measurements were carried out at an angle of incidence of 75°.

RESULTS AND DISCUSSION

The development of imaging ellipsometry, which combines the power of ellipsometry with microscopy, potentially expands ellipsometry into new areas of biological analysis. The potential applications of an immunosensor based on imaging ellipsometry were demonstrated here with three examples. The first, one kind of simple bioprobe, was used to detect antigen/antibody interaction. The second, a protein chip with four kinds of proteins, was presented for multi-protein detection. The third, multiprocess of protein interactions, was monitored by the immunosensor in real time.

Bioprobe. The probe coated with BSAu was used to detect anti-BSA in serum. The result of an image in gray scale format is shown in Figure 3A. There were three regions with different gray scales on the probe. The dark region was the substrate with a hydrophobic layer of dichlordimethylsilane. The middle region was the saturated BSA layer immobilized on the hydrophobic surface. The bright region was the saturated BSA/anti-BSA complex layer introduced by anti-BSA molecules in serum binding with BSA molecules immobilized on the surface. The intensity I of layers in Figure 3A was proportional to the square of the thickness of the layers, so the different gray scales of layers indicated different thickness of layers. The higher the intensity of the image, the higher the thickness. The thickness of BSA/anti-BSA complex layer was obviously higher than that of the BSA layer. The absolute thicknesses of layers in Figure 3 were calibrated with a conventional ellipsometer, and the surface concentrations were deduced from the thicknesses, BSA/anti-BSA complex concentration 0.744 μg/cm^2 and BSA 0.252 μg/cm^2. The image in gray scale format could be easily deduced into a thickness distribution in three-dimensions according to the relationship between the intensity and the thickness, which is shown in Figure 3B.

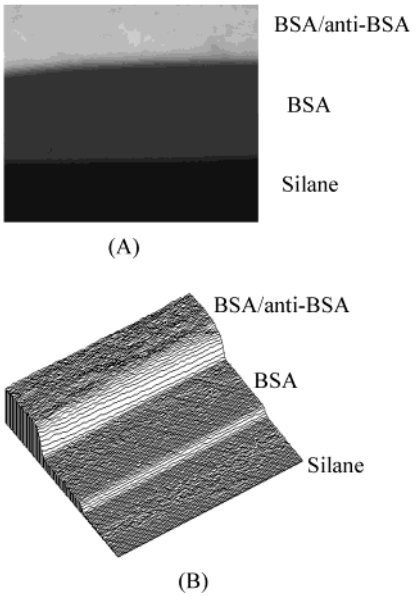

Figure 3. Immunosensor based on imaging ellipsometry for the detection of anti-BSA. (A) Image in gray scale format of the saturated BSA sensing layer and the saturated BSA/anti-BSA complex layer on the silane layer immobilized on the silicon substrate. From the bottom to the top, the layers in the image are silane layer, the saturated BSA layer, and the saturated BSA/anti-BSA complex layer. (B) Image in three dimensions deduced from (A) according to the principle that the intensity in image is proportional to the square of the thin-layer thickness.

The field of view of the immunosensor was large enough to view all layers of the probe simultaneously, typically within 1 s. It was a faster and more convenient method to compare the difference of surface concentration between layers in qualitative fashion, rather than conventional ellipsometry, which measured only layer by layer with time. In terms of quantitative measurement, the average intensity of the protein layer was obtained easily by averaging all pixels on the whole layer at the same time with the immunosensor. For a conventional ellipsometer, it has to measure several spots on the protein layer and then get an average thickness. In addition, the obvious contaminated areas on the layer could be excluded to make the quantitative data more reliable with the immunosensor.

In biological research, clinical diagnosis or bioindustries, especially relating to proteins, the concentration or bioactivity often needs to be detected. Many methods are used currently, such as Biuret, enzyme-linked immunoadsorbent assay, radioimmunoassay, liquid chromatography, and electrophoresis. The immunosensor can serve as a convenient alternative method to detect protein concentration or bioactivity. Samples can be detected directly without any labeling, which avoids disturbances from conjugated markers or handling of radioactive materials. The

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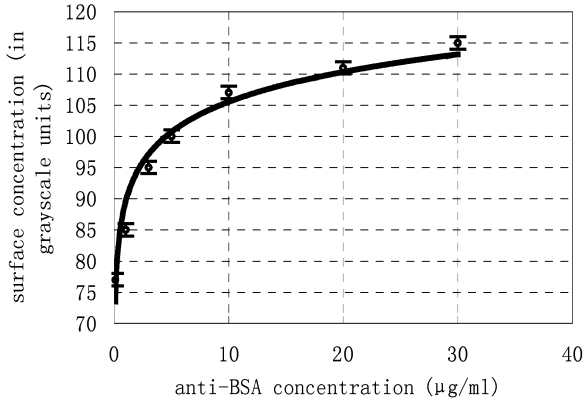

Figure 4. Calibration curve for anti-BSA concentration detection. The incubation time is 30 min.

detection time can be largely reduced without the time consumed for protein labeling. The assay with the immunosensor relies on a natural functional interaction, such as the interaction between antigen and its antibody; therefore, the active concentration of antibody is measured instead of the total antibody. The typical assay procedure with the immunosensor is like any other assay: optimize assay conditions for the desired range of analyte concentrations, establish a calibration curve using known concentrations of analyte to cover the required range, and measure the unknown concentration of analyte. The calibration curve shown in Figure 4 was established to measure the concentration of anti-BSA in serum. The concentration range was from 0.1 to 30 μg/mL. The incubation time was 30 min, and the data were obtained by averaging three replicate experiments. The result is shown here only for demonstration purposes. In a real application, the relevant protein should be selected, the proper concentration range should be chosen, and detection conditions should be optimized. In terms of sensitivity, reported detection limits were 0.1 ng/mL hepatitis B surface antigen, 0.01 ng/mL α-fetoprotein, and 10 amol/mL in DNA hybridization studies by ellipsometry.[7]

Protein Chip. A protein chip was used to detect multianalytes simultaneously in solution. Eight analytical areas of a protein chip were chosen to immobilize proteins on them (shown in Figure 5A). The result of proteins immobilization in three-dimensional format image was shown in Figure 5B. There were 10 analytical areas and 4 kinds of proteins immobilized in duplicate on these analytical areas individually, which were BSA, Fib, IgG, and HSA. The left two analytical areas were used as references. The intensities of protein layers were different. The average intensities in gray scale of protein layers are listed in Table 1.

The protein chip was incubated with a mixture of antiserum, which contained anti-IgG, anti-HSA, and anti-Fib. Antibodies in solution bound with corresponding antigens immobilized on the protein chip to form complexes, and the surface concentrations on analytical areas where the antibody/antigen interactions took place increased. As expected, the surface concentrations on the analytical areas immobilized with Fib, IgG, and HSA increased. The result was detected with the immunosensor and is shown in Figure 5C. The average intensities in gray scale of protein layers are listed in Table 1.

For demonstration purposes, only a few spots are shown here. In fact, the area of each spot can reduced to fabricate more spots on a silicon surface. The area of each spot in Figure 5 is 0.75 × 0.75 mm^2, and the lateral resolution of the immunosensor is 3 μm, so there is much room to reduce the area of each spot. In this paper, the protein solution was dropped on the protein chip by pipet manually for only a few spots. To drop large numbers of spots on a patterned silicon surface, a pipetting robot with microfluidics and micropump controlled by computer had to be used.

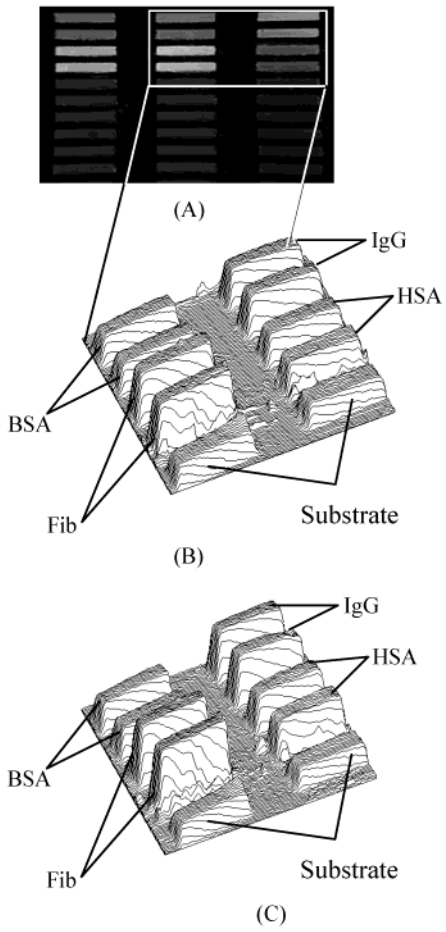

Figure 5. Immunosensor based on imaging ellipsometry for detection of anti-Fib, antiIgG, and antiHSA protein chip. (A) Image of the protein chip in gray scale format. (B) Three-dimensional image of protein chip with ligands immobilized in duplicate: BSA, Fib, IgG, and HSA, respectively. (C) Three-dimensional image of the protein chip after incubation with antisera solution containing anti-Fib, anti-IgG, and anti-HSA.

Table 1. Average Surface Concentration (in Gray Scale Units with an Error of ~±2) in IgG Areas, HSA Areas, Fib Areas, and BSA Areas before and after Incubation in a Mixture of Antiserum

	before incubation	after incubation
IgG area 1	68	124
IgG area 2	71	125
HSA area 1	58	91
HSA area 2	55	92
Fib area 1	108	191
Fib area 2	109	190
BSA area 1	68	70
BSA area 2	67	69

The protein chip is a potentially powerful tool in biochemistry and molecular biology.[13] This field is expanding rapidly now, and many types of chips have been designed,[14−17] but most of them are analogous with DNA chips in terms of fabrication and detection. Proteins are labeled with fluorescence or enzyme and the results are detected with readers. Kodadek pointed out several problems concerning protein labeling in the literature.[13] First, the chemical heterogeneity of proteins makes this hopeless as a strategy for doing quantitative work. Some proteins will be labeled far more efficiently than others. Then the chemical labeling of proteins can change their surface characteristics greatly, which can result in significant protein denaturation, especially for smaller proteins and peptide hormones. Finally, protein labeling is a labor-intensive processing step for a high-throughput protein chip. As a label-free and multisensor detection method, the immunosensor based on imaging ellipsometry is a promising technique in the protein chip area.

Real-Time Monitoring for Protein Interactions. The immunosensor based on imaging ellipsometry not only was used for ex situ detection as mentioned above but also offered scientists a powerful tool for monitoring protein interaction process in situ and in real time to provide protein interaction kinetics information. Combining with the protein chip, the immunosensor can detect the growth process of the multilayer during the label-free binding processes simultaneously. To show the real-time analysis with the immunosensor, a chip with protein Fib, HSA, and IgG immobilized was prepared. The chip was inserted into the reaction cell, and the mixture of antiserum containing anti-Fib, anti-HSA, and anti-IgG was poured into the cell. Several binding processes between antibodies in solution and antigens immobilized on the surface were recorded with a series of images in gray scale format via time simultaneously by the immunosensor. The surface concentrations of layers on analytical areas of each image were measured and plotted versus time to get the binding curves. The binding curves of IgG/anti-IgG, Fib/anti-Fib, and HSA/anti-HSA are shown in Figure 6. The reaction between immobilized antigens and antibodies in solution can be assumed to follow pseudo-first-order kinetics as described in the literature,[18−21] and the association rate constants or dissociation rate constants can be obtained by an analogous analysis method. The detailed data process and kinetics analysis will be presented in another paper.

The ability of multisensing of the immunosensor based on imaging ellipsometry makes it potentially suited for monitoring the multiprocess of protein interactions. The most obvious application is the multianalyte chips wherein more ligands, each responsive to a different analyte, are monitored to offer kinetics information just like the example above. The more important application is to monitor cross-reactive interactions simultaneously. A cross-reactive chip contains many immobilized ligands that bind to the same analyte but with different binding affinity. The combination between the immunosensor and the cross-reactive chip can offer a convenience to compare these ligands' binding affinity under the same conditions, and ligands with high affinity will be screened.

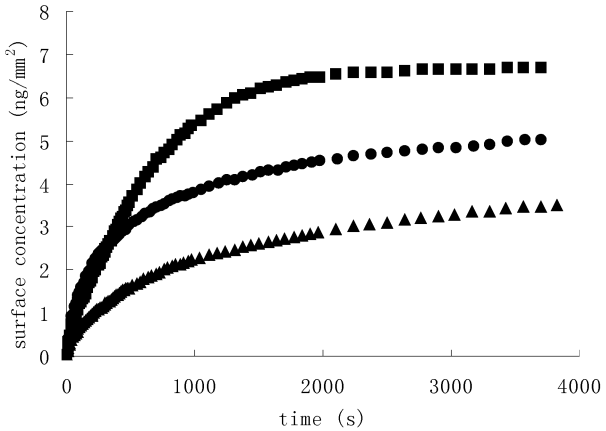

Figure 6. Binding curves of anti-Fib/Fib (■), anti-IgG/IgG (●), and anti-HSA/HSA (▲) obtained by the immunosensor based on imaging ellipsometry. Each antibody concentration was 25 μg/mL.

CONCLUSIONS

An immunosensor based on imaging ellipsometry and its potential applications have been demonstrated in this study. It was shown that the immunosensor is a fast, reliable, and convenient technique to measure protein surface concentration or detect protein concentration in solution. The immunosensor has a large field of view that makes it as a multisensor to detect multiple analytes simultaneously with the protein chip. As a nondestructive and fast optical detection technique, the immunosensor can monitor multiple binding processes simultaneously in situ and in real time. Another advantage of the immunosensor is to detect samples without any labeling, which avoids disturbance from conjugated markers or handling with radioactive materials and largely simplifies the detection operation. This allows the application in a broad range of different biological systems. These advantages make the immunosensor potentially suited as a promising high-throughput screening technique.

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