Real time detection of antibody–antigen interaction using a laser scanning confocal imaging-surface plasmon resonance system*

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(Received 22 July 2011; revised manuscript received 13 August 2011)

A laser scanning confocal imaging-surface plasmon resonance (LSCI-SPR) instrument integrated with a wavelength-dependent surface plasmon resonance (SPR) sensor and a laser scanning confocal microscopy (LSCM) is built to detect the bonding process of human IgG and fluorescent-labeled affinity purified antibodies in real time. The shifts of resonant wavelength at different reaction time stages are obtained by SPR, corresponding well with the changes of the fluorescence intensity collected by using LSCM. The instrument shows the merits of the combination and complementation of the SPR and LSCM, with such advantages as quantificational analysis, high spatial resolution and real time monitor, which are of great importance for practical applications in biosensor and life science.

Keywords: laser scanning confocal imaging-surface plasmon resonance, combination, complementation, real time

PACS: 06.90.+v, 07.60.–j, 42.30.–d, 87.15.km **DOI:** 10.1088/1674-1056/21/2/020601

1. Introduction

Surface plasmon resonance (SPR) sensors have been widely investigated as an exceedingly powerful tool for biological and chemical detection in the past decade.[1−5] The highly sensitive optical reflectivity of the gold to the change of dielectric environment provides the SPR sensor with a great means for detecting the contaminants in water, food, air and label-free biochemical assays.[6−10] SPR sensing has been demonstrated to be a great valuable technique to identify the interaction process of biomolecules (such as antigen–antibody) and to quantify their equilibrium constants, kinetic constants and underlying energetics to understand the immunifaction well.[11,12] During the SPR sensing, the interaction process is deduced from the changes of optical response based on the resonance angle or wavelength. However, the results of the SPR are the average effect of the testing region. Distinguishing the local interaction processes *in situ* related to the SPR signals is still lacking experimentally in the study of such subjects as the distribution of nanomonomer arrangement, movement and energy transfer, signal transduction for biological molecules and proteins in cells.

As a new generation of high-resolution methods of analysis, laser scanning confocal microscopy (LSCM) has been extensively applied in many areas of current research interest, such as biology,[13−15] materials,[16,17] nano techniques,[18,19] etc. It offers several advantages over conventional optical microscopy in several respects, including the controllable depth of field, the elimination of image degrading out-of-focus information and the ability to collect serial optical sections from thick specimens.[20] There has been a tremendous explosion in the popularity of LSCM in recent years, due in part to being relatively easy to obtain extremely-high-quality images

*Project supported by the Instrument Developing Project of the Chinese Academy of Sciences (Grant No. YZ200740), the National Natural Science Foundation of China (Grant Nos. 60978034 and 10974019), and the National High Technology Research and Development Program of China (Grant No. 2009AA03Z318).

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from specimens. But most LSCM work is qualitative analysis and it is very hard to achieve quantitative detection directly.

In the present study, a laser scanning confocal imaging-surface plasmon resonance (LSCI-SPR) instrument is developed by integrating a wavelength-dependent SPR with an LSCM. By combining the virtues of the two techniques, this instrument may be used successfully to monitor the process of antibody–antigen interaction in real-time and *in situ*. The time-dependent intensity of the fluorescence of antibody fits well with the change of the resonance wavelength in the antibody–antigen interaction process. The technique extends the functions of the conventional SPR sensor and provides the real-time and *in-situ* detection of the biomolecules.

2. LSCI-SPR system and experiment

The schematic diagram of our experimental setup for SPR combined with LSCM is shown in Fig. 1. The wavelength-dependent SPR sensor is in a custom-built style. White light from an LED (HLV-24SW-3W, Japan) was collimated, polarized in TM mode (TM-polarization) and coupled to the sample film via a BK7 prism. The reflected light was collected into a fiber spectrometer (AH-ISW301, China) equipped with a photomultiplier tube. The incidence angle was varied freely by adjusting the rotation arms on the instrument by two rotary stages (KS432-75, Japan) and the resonance minimum at a given wavelength was positioned accordingly. The biosensor substrates were prepared by sputtering 2-nm Cr and 50-nm Au onto BK7 slides. The side of the slide with Au film was immersed in the flow cell and the other blank side was attached to the prism using refractive index matching liquid. The prism, the substrate and the flow cell were fixed on the stage of the inversed microscope of the LSCM (Nikon C1 Si, Japan) system. The 40× objective (Nikon CFI Plan Flour ELWD DM N.A. 0.6 W.D. 3.7 mm–2.7 mm) was employed to collect the fluorescence image of the fluorescein-labeled affinity purified antibody with the excitation at 488 nm by a multi-line Ar laser. The focal plane was positioned on the gold film, so that the fluorescence image could be detected just when the antibody interacted with the antigen. The fluorescence of the antibody in the flow cell was too weak to be recorded. The samples were injected into the flow cell by a spring pump (Harvard 33 Twin Syringe Pump, USA).

In our experiment, human normal IgG and affinity purified antibody isolated from a pool of serum from goats immunized with purified human IgG labeled by reaction with fluorescein isothiocyanate (FITC) were obtained from Beijing Seajet Scientific Inc. The 3-mercaptopropionic acid (MPA) was purchased from Sigma (St. Louis, MO, USA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) and bovine serum albumin (BSA) were obtained from Huamei Biotechnology Co, China. All the biological reagents were kept in refrigerator at 4 °C. All other chemicals were of analytical-reagent grade. All solutions were prepared with ultrapure water (18.4 MΩ·cm^{-1}, Millipore Corp.). The 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4) and 0.3 mol/L citrate buffer (pH 2.7) were prepared.

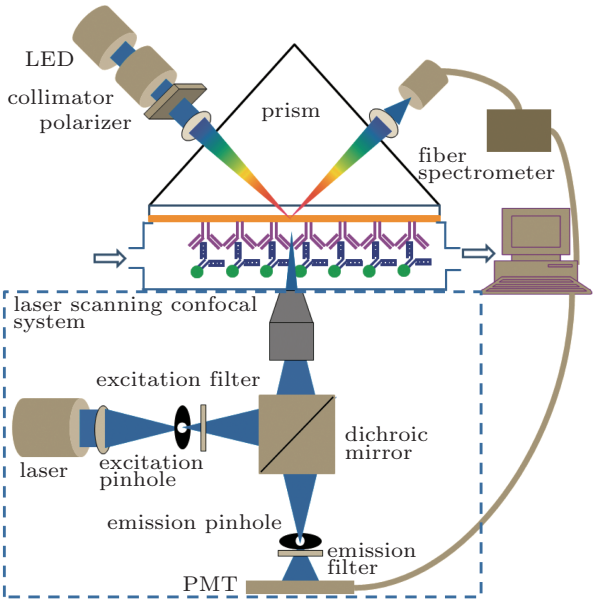


Fig. 1. (colour online) Scheme of the LSCI-SPR instrument.

The biosensor substrates were rinsed with ultrapure water, treated with ethanol for 5 min and rinsed with ultrapure water again. The substrates was soaked in the 10-mmol/L MPA solution for 12 h for full reaction, because the sulfide bond of MPA can stick firmly on the gold surface to form a self-assembly monolayer; then it was activated by injecting a solution containing NHS (100 mg/mL) and EDC (100 mg/mL) for 10 min. In this process, the –COOH group reacted with NHS and NHS reacted with MPA under the catalysis of EDC. The remaining EDC and NHS were removed from the sensor surface by injecting PBS buffer. The human IgG was injected into

the flow cell to be assembled on the activated surface with 1 μL/min reactive rate. The assembling of the human IgG was carried out for 1 h to organize the processing antibody on the solid surface and form a stable biosensor membrane. The BSA was injected into the flow cell in order to react with the remaining MPA for 2 min. Then the PBS buffer was used to wash off the non-covalently bound antibody to stabilize the baseline. The fluorescein-labeled, affinity purified antibody was injected into the flow cell at a rate of 500 μL/min and bonded to the human IgG. The immunocomplex on the biosensor surface can be dissociated with 0.1 mol/L PBS for 10 min and 0.3 mol/L citrate buffer (pH 2.7) for 40 min. The regenerated biosensor surface can be reused. The interaction process was monitored in real-time using the LSCI-SPR simultaneously at room temperature.

Each step of the reaction during surface modification, antibody immobilization and substrate regeneration was monitored using SPR. The resonant wavelengths of SPR were eventually constant with the increase in process time, suggesting the complete reactions. However, in this report, we just focus our attention on the bonding of human IgG and fluorescein-labeled affinity purified antibody. Not all the SPR data are shown here.

3. Results and discussion

We recorded the SPR curves during the bonding process of human IgG and fluorescein-labeled antibody at a 3-min interval; meanwhile the fluorescence images were collected in real-time. The concentration of human IgG was fixed at 500 μg/mL and the concentrations of antibodies were set at different values of 1 μg/mL, 5 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL to verify the bonding process sequentially. Figure 2 shows the selected shift of resonant wavelengths during a typical bonding process of human IgG and antibody with concentrations of 500 μg/mL and 5 μg/mL, respectively (not all of the curves are shown here). The initial resonant wavelength λ_1 is about 624 nm, as the baseline and finally the maximum wavelength λ_2 is about 628 nm, indicating the fully reaction. Once a determination was performed, the biosensor surface would be treated using PBS for 10 min and then 0.3 mol/L citrate buffer (pH 2.7) to regenerate it for 40 min. The resonant wavelength reached the baseline again.

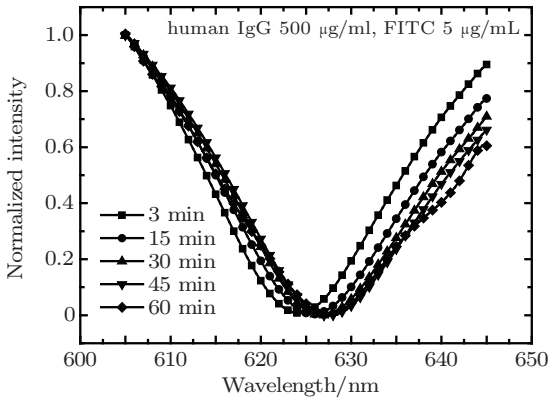


Fig. 2. Reflection spectra of a typical bonding process of human IgG and antibody with concentrations of 500 μg/mL and 5 μg/mL, respectively, at different time stages.

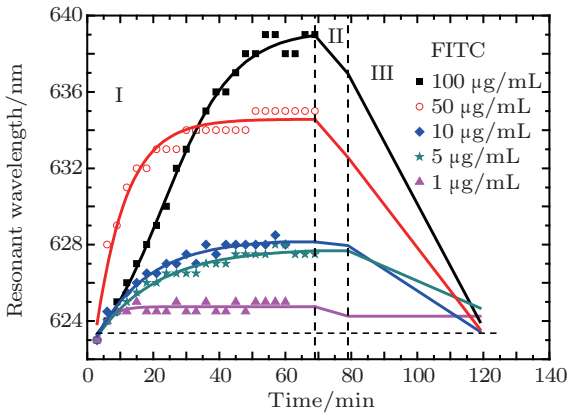


Fig. 3. (colour online) Reaction and regeneration processes for human IgG and antibody with different concentrations.

All of the SPR data from the reaction process for human IgG and antibody with different concentrations are recorded in Fig. 3. The data of the resonant wavelength was obtained from each SPR spectrum for different reaction times. The solid lines are for guidance to the eyes. Region I shows the bonding process for human IgG and antibody. The obvious shifts of the resonant wavelength indicate that the refractive index changes with the reaction process going on and the flat states were obtained when the reactions were finally finished. Different concentrations of antibodies indicate different reaction rates and maximum shifts of resonant wavelength. The maximum shift $\Delta\lambda$ reached about 15 nm for the antibody with 100 μg/mL concentration. Regions II and III show the processing of the fluorescein-labeled affinity purified antibody dissociated from human IgG MPA surface by PBS and citrate buffer washing separately. The LSCM was used simultaneously to collect the fluorescence image in real time, to monitor the reaction processing. A typical fluorescence image sequence and

intensity for the FITC concentration 50 μg/mL was presented in Fig. 4.

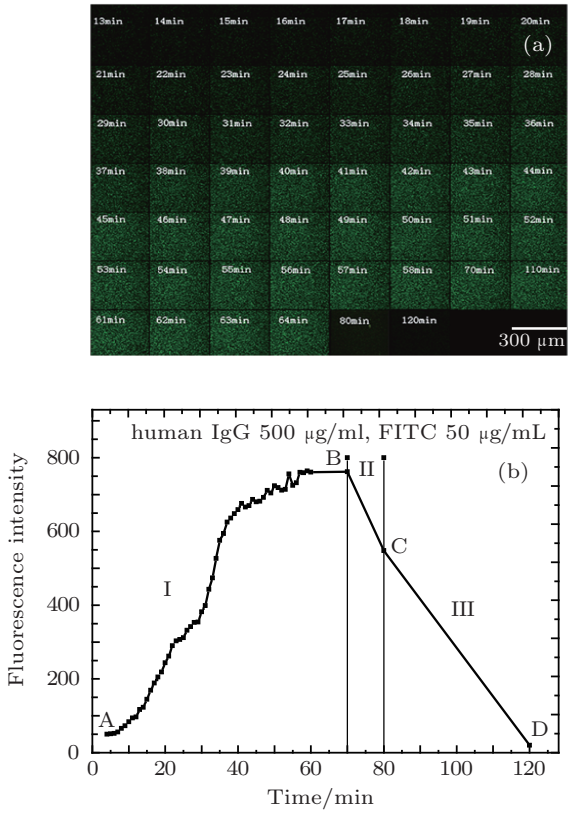


Fig. 4. (colour online) Typical fluorescence images of the reaction and regeneration processes for IgG (500 μg/mL) and antibody (50 μg/mL) collected by LSCM, where panel (a) shows the fluorescence images with time sequence; panel (b) exhibits the intensity of the fluorescence images.

Each image was captured at a 1-min interval (Fig. 4(a)), almost corresponding to the SPR data at the same time. As mentioned above, the focal plane was fixed on the surface of the gold film or the human IgG single layer plane. So the fluorescence images could be collected only when the fluorescent-labeled antibody was bonded to the human IgG. The change of the fluorescence intensity of the images within 10 μm×10 μm area (Fig. 4(b)) is corresponding to that of SPR data shown in Fig. 3 (The line with the circle points). These results indicate that the interaction can be *in situ* recorded by images in real time. The concentration of the free fluorescent-labeled antibodies in the flow cell (not in the focal plane) was too low to be detected using the microscopy. The trend of the intensity of the fluorescence corresponds well to the shift of the resonant wavelength SPR wavelength. At the same time, the three-dimensional (3D) images can show the local information at different times (Fig. 5). The 3D images at times A, B, C and D with the interaction process fluorescence intensity defined in Fig. 4(b) are shown in Figs. 5(a)–5(d) respectively. It offers us a direct way to observe the reaction process of biomolecules especially with the detailed information about the local interesting sites.

The LSCI-SPR biosensor can also be used to analyse the reaction process quantitatively. The association and the dissociation rate constants $K_{\rm a}$ and $K_{\rm d}$ in a flow cell for a 1:1 interaction can be determined via the standard equation as follows:

$$\mathrm{d}\Delta\lambda/\mathrm{d}t = K_a C(\Delta\lambda_{\max} - \Delta\lambda)K_{\rm d}\Delta\lambda, \qquad (1)$$

where $\mathrm{d}\Delta\lambda/\mathrm{d}t$ is the rate change of SPR response, C is the concentration of analytes, $\Delta\lambda_{\max}$ is the maximum analyte binding capacity and $\Delta\lambda$ is the shift of the resonance wavelength. The further detailed study is not involved in this paper. Although the fluorescence of the LSCM can only give qualitative analysis during the reaction process, it is worth while noting that the LSCM possesses a powerful spatial resolution. The SPR measurement could be expected to be performed in a particular local area via the LSCM. Therefore, the LSCM-SPR instrument with the functions of real time monitor and quantificational analysis in local interesting sited has further potential applications in biosensor and life science.

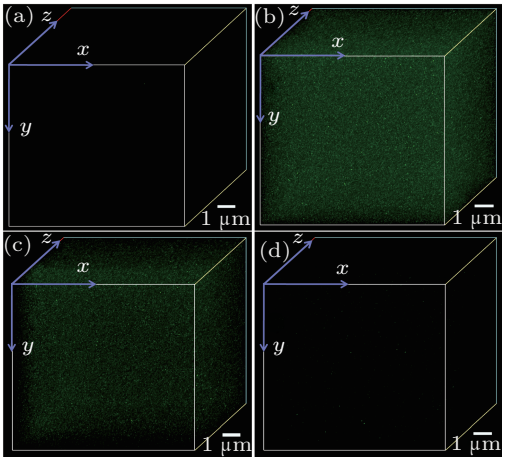


Fig. 5. (colour online) The 3D fluorescence images for times A, B, C, and D with the interaction process fluorescence intensity defined in Fig. 4(b).

4. Conclusion

The bonding process of human IgG and fluorescein-labeled affinity purified antibody is monitored in real time using the LSCI-SPR instrument. The shifts of resonant wavelength at different reaction

time stages obtained by SPR correspond well with the change of the fluorescence intensity collected by using LSCM. The LSCI-SPR instrument shows the merits of the combination and complementation of the two techniques, which are of great importance for practical applications in biosensor and life science due to its quantitative analysis, high spatial resolution and real time monitor.

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