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# Detection of SARS-CoV Antigen via SPR Analytical Systems with Reference

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## 1. Introduction

Surface Plasmon Resonance (SPR) is an extremely sensitive optical technique to detect the changes in refractive index occurring at the metal interface, which can monitor the processes in real time without labelling requirements (Lee et al., 2006; Kanda et al., 2004; Homola, 2003). Since 1989, our group have been focusing on SPR technology development. Several generations of SPR analytical platforms, including Reference SPR Analysis System, Electrochemical-SPR Analysis System, Portable SPR Analysis System, and High-Throughput, Multi-analyte Imaging SPR Analysis System, have been designed, fabricated and tested for various applications.

SARS (Severe Acute Respiratory Syndrome) is a kind of serious infection, which caused tremendous loss in China at 2003. It is caused by the infection with SARS-associated coronavirus (SARS-CoV) (Rota et al, 2003) and early diagnosis of SARS is very important for better control of future SARS epidemics (Drosten et al, 2003). There have been several diagnostic methods currently used in hospital, but none of them can be used for early detection with low cost. For example, ELISA cannot detect antibodies that were produced in the infected patients within the first two-week's infections..

In this chapter, a novel detection method of the SARS-CoV antigens was reported by using a home-developed Reference SPR Instrument, which can monitor reaction spots with control in a single flow cell. Monoclonal antibodies to SARS-CoV were immobilized only on the reaction spots via selective chemical modification, with no immobilization on the reference spots. Then a higher version of reference SPR analytical system: high-throughput, multi-analyte imaging SPR (HMI-SPR) analytical system, was developed and tested successfully

## 2. Theory

Surface plasma resonance (SPR) is a physical phenomenon which occurs when a polarized light beam is projected through a prism onto a thin metal film (gold or silver). At a specific angle of the projected light, resonance coupling between light photons and surface plasmons of the gold can occur when their frequencies match. Because the resonance leads to an energy transfer, the reflected light shows a sharp intensity drop at the angle where SPR takes place. Resonance coupling of the plasmons generates an evanescent wave that extends 100 nm above and below the metal surface. As an analytical tool it is important that a

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change in the refractive index within the environment of the evanescent wave causes a change of the resonant angle, when the sharp intensity drop can be observed. Binding of single target biomolecule to immobilize single molecule on top of the metal (gold) surface of the sensor leads to a change of refractive index and could be recorded as a change of intensity in the reflected light by a detector. This setup enables real-time measurement of biomolecular interactions, with refractive index changes proportional to mass changes.

Generally the Kretschmann configuration and resonance angle modulation are used in SPR. Kretschmann configuration (Kretschmann & Reather, 1968) is the most-common geometry setup of SPR platforms, in which the incident light comes from the high refractive index medium (prism) and reflects at the gold surface without travelling through the liquid. The resonance angle is defined according to the resonance peak of SPR, which is further defined based on the SPR curve, obtained by recording the change of light intensity of the reflective light along with the change of angle of the incident light. The resonance peak of SPR is at the lowest point of the SPR curve, where light absorption is the maximum and the light intensity of the reflective light is the minimum under a specific angle of the SPR curve.

SPR is a well established technique for observing biomolecular binding reactions. In a usual setup, light traveling through a high refractive index (RI) substrate reflects from the substrate surface, which is coated with a thin layer of gold (50 nm). Certain biomolecules have been immobilized on the gold surface, which bind the targeted biomolecules in aqueous samples. As targeted biomolecules bind to the gold surface, the surface RI increases roughly proportional to the quantity of the molecules immobilized. The change of the surface RI results in the shift of the resonance peak of SPR and detection of the shift of the resonance peak of SPR monitors the reaction between biomolecules quantitatively such as the concentration of targeted molecules. Since the shift of the resonance peak of SPR could also be brought by other disturbances (e.g. temperature changes), it is necessary to use control signals to distinguish the shifts caused by the reactions from the shifts by other interferential factors.

### 3. Experimental

#### 3.1 Reagents and materials

Protein A (from *Staphylococcus aureus*), EDC, NHS, HEPES and ethanolamine were purchased from SIGMA company. Human Immunoglobulin G (IgG), rabbit IgG, goat anti human IgG, and goat anti rabbit IgG were purchased from Beijing Xijingke Biotechnology Company.

Sterilized SARS-CoV (PUMC-01 strain, TCID<sub>50</sub> is 10<sup>6</sup>puf/ml) and monoclonal antibodies (11G9, 2F1, 13D10, 11H12) to this virus were supplied by the Institute of Laboratory Animal Science, CAMS & PUMC.

The others were purchased from Beijing Chemical Reagents Company.

#### 3.2 Bare gold slide patterning

The patterns of bare gold slides with reaction areas and reference areas were designed in L-edit and transferred to a photolithograph mask of chromium. The fabrication was as follows. The glass slides were spin coated with positive photoresist (AZ 1500) and patterned by an MA4 optical stepper (Karluss, Germany). The exposed photoresist was developed and the glass slides were coated with 2 nm of chromium plus 50 nm of gold in a JGP560B3 type magnetron sputtering instrument (SDY Technology Development Co., LTD, Chinese

Academy of Sciences, China). After removing photoresist in acetone, the bare gold slides with patterned areas were finally obtained (Fig.1). Prior to each experiment, bare gold slides were washed in ethanol to remove fingerprints, oily residues and dust particles.

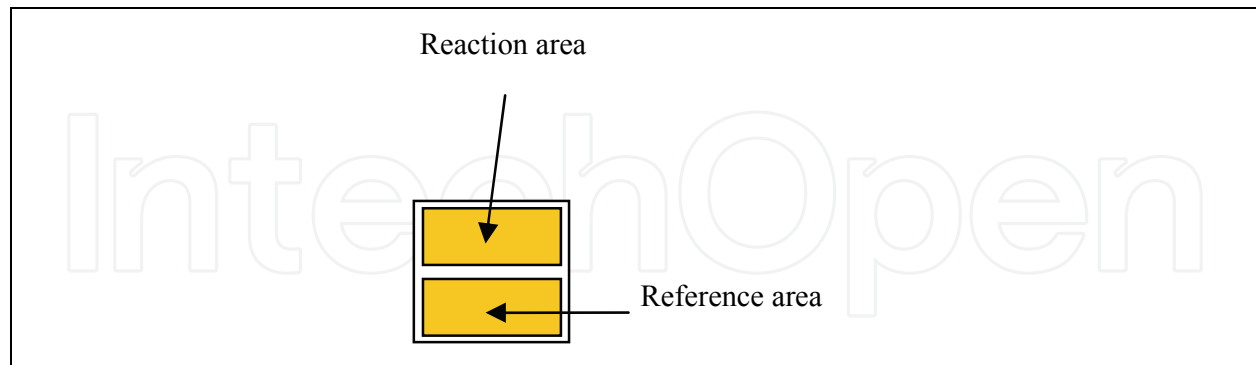


Fig. 1. Schematic of a SPR biochip with a reference area and a reaction area

### 3.3 SPR chip preparation

The sensor chips were prepared in the following procedure. Firstly bare gold slides were treated in piranha solution ( $(\text{H}_2\text{O}_2\ 35\%): (\text{H}_2\text{SO}_4\ 96\%) = 3:7$ ) at  $90^\circ\text{C}$  for an hour. After the slides were washed by deionized water and ethanol, they were soaked in the 16-mercapto-hexadecan-1-ol solution ( $5 \times 10^{-3}\text{mol/L}$  16-mercapto-hexadecan-1-ol in 80% ethanol and 20% water) to obtain a hydrophilic surface. Then the slides were soaked in the solution including  $0.4\text{mol/L}$  sodium hydroxide, diglyme and  $0.6\text{mol/L}$  epichlorophdrin at  $25^\circ\text{C}$  for 4h. After the slides were thoroughly washed by deionized water, ethanol and deionized water sequentially, they were soaked in the dextran solution ( $3.0\text{g}$  dextran T500 in  $10\text{ml}$   $0.1\text{mol/L}$  NaOH) at  $25^\circ\text{C}$  for 20h. Finally, a bromoacetic acid solution was only dropped on the detection gold areas of the slides at  $25^\circ\text{C}$  for 16h. A thin layer of carboxymethyl dextran was formed only on the detection gold areas of the slides, while nothing was immobilized on the reference gold areas.

### 3.4 Protein immobilization online

After a SPR chip was mounted in the home-developed Reference SPR Analytical System, the angle scanning was conducted to choose a proper position for the fixing angle detection. HBS buffer (HEPES,  $0.01\text{mol/L}$ ; NaCl,  $0.15\text{mol/L}$ ; Tween 20, 0.05%, pH7.4) was firstly introduced to wash the chip. Then the mixed solution of EDC and NHS (final concentrations were  $0.2\text{mol/L}$  and  $0.05\text{mol/L}$ , respectively) was used to activate the SPR chip.

After activation, the chip was washed with HBS buffer. Then a protein solution (pH of the solution is less than PI of the protein) was introduced into the chip to react with the activated area for protein immobilization.

In order to eliminate the activated sites without immobilizing proteins, the chip was deactivated by flushing in Ethanolamine ( $1\text{M}$ ) to remove molecules adsorbed loosely on the chip (Johnsson et al, 1991).

### 3.5 SPR instrument setup

As shown in Fig. 2, a Reference SPR Instrument System, with the function of qualitative and quantitative measurements, was manufactured by our group. The reaction area and the

control area of the system could be simultaneously detected in one flow channel by using this new Reference SPR Instrument System, resulting in recording two signals at one run in which one signal shows the curve of the reaction, and the other shows the curve of reference simultaneously. The range of the angle adjustment is  $40^{\circ}\sim 70^{\circ}$ , which makes it feasible for both the gas sample and liquid sample measurements. The detection resolution of the resonance angle is  $0.001^{\circ}$ . And the detection range of refractive index is from 1.04 to 1.43 with a sensitivity better than  $1\times 10^{-5}$ RIU.

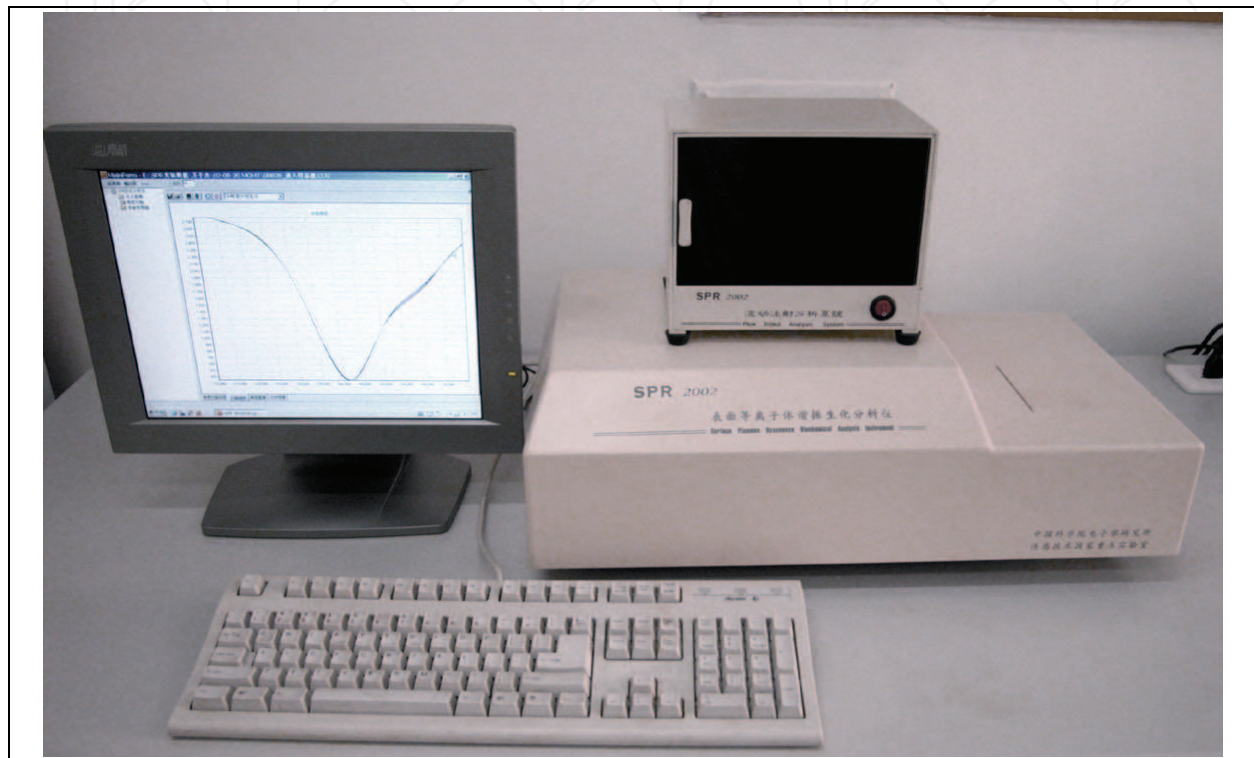


Fig. 2. Photograph of the home-developed Reference SPR Instrument System

## 4. Results and discussion

### 4.1 Design of home-developed reference SPR analytical system

As shown in Fig. 3, the reference SPR analytical system consists of a semiconductor laser (1), a system of a polarizing filter and lens (2), a prism (3), a high resolution detector (4), a computer (5), a sampling system (6), a SPR chip with reference (7) and a microfluidic device with a microchannel (8). The laser and the optical system were installed on a circumrotated arm, while the high-resolution detector was installed on another circumrotated arm. The prism, the SPR chip and the microfluidic device were installed on an anti-floating surface between the two circumrotated arms. Both circumrotated arms were controlled by the stepper motor with the coding system. During experiments, a beam of the light from the semiconductor laser with the wavelength of 650nm through the optical system shined in the prism and reached the SPR chip. Then the beam of the light reflected into the detector. Finally, the intensity of the reflected light was recorded and exported to the computer. The sampling system controlled two peristaltic pumps and different solutions were introduced to the SPR chip by using the microfluidic device.



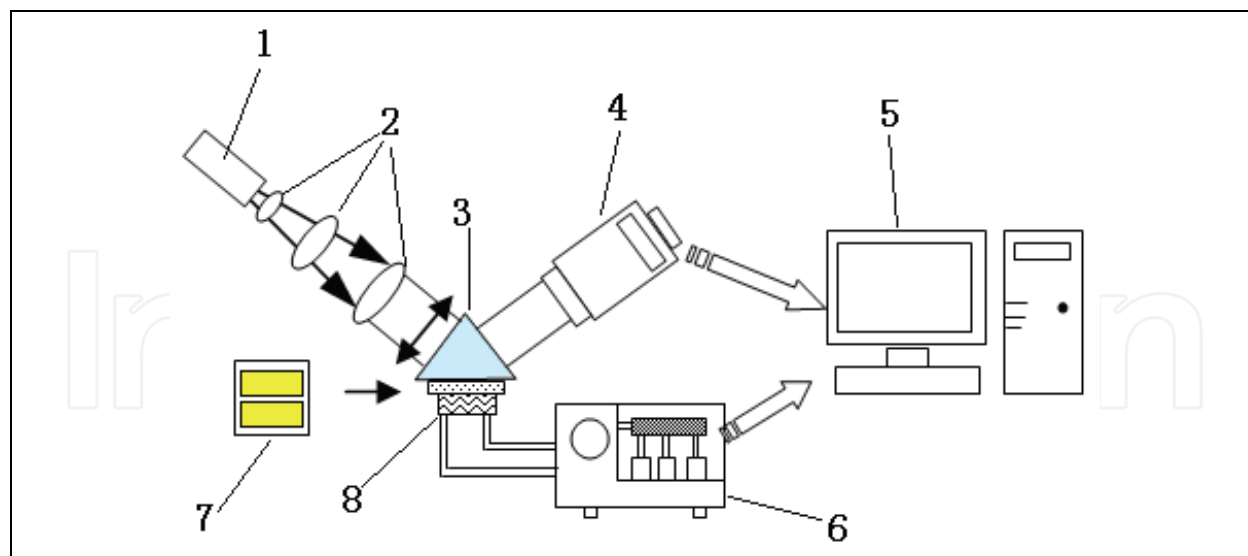


Fig. 3. Schematic of the home-developed Reference SPR Instrument System

**4.2 Detection of SARS-CoV by immobilizing antibodies directly**

In the type I design, the antibodies to SARS-CoV were immobilized directly on the experiment area after the chip was activated. The solution of the monoclonal antibodies to SARS-CoV (pH 4.6) was pumped into the microfluidic device for 2000s. Then ethanolamine (1M, pH 8.5) was pumped in to deactivate the chip for 10 min. Finally HBS buffer was pumped in to wash the SPR chip. The signal recording of the immobilization step is shown in Fig. 4. When introducing the monoclonal antibodies to SARS-CoV, the reaction line increased significantly and the light intensity of the reaction line after the deactivation was still higher than the intensity before the antibody immobilization. This light intensity increase of the reaction line with reference line unchanged demonstrated that the monoclonal antibodies to SARS-CoV were successfully immobilized on the reactive area.

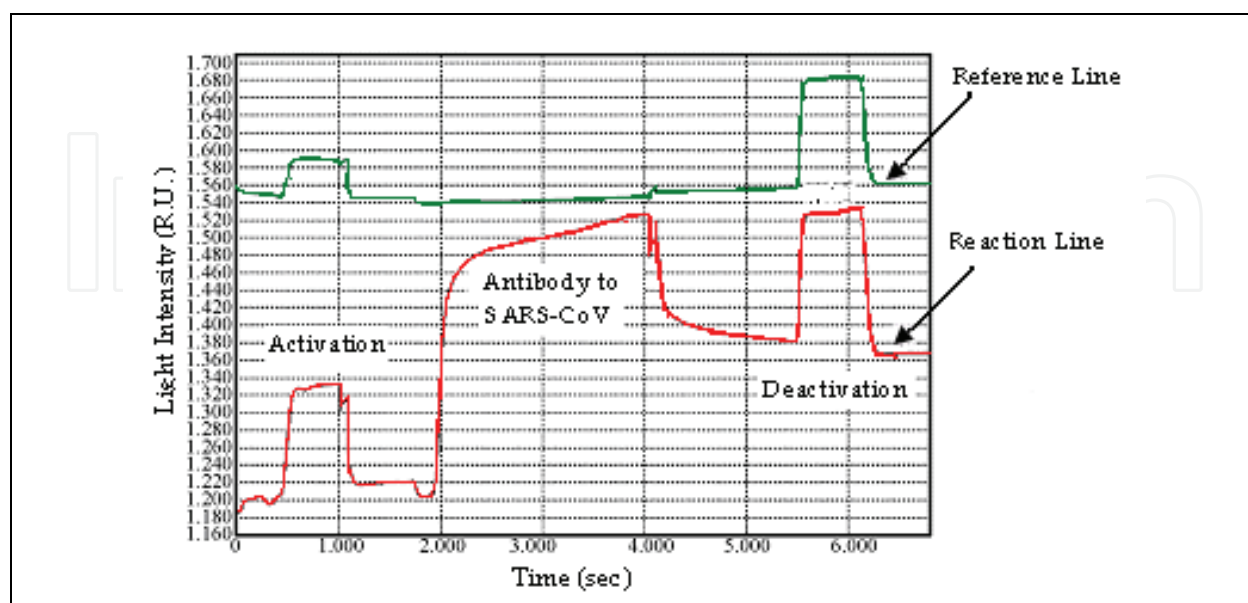


Fig. 4. Signal recording of immobilizing antibodies directly  
The green line is a reference curve. The red line is a reaction curve.

After the antibodies were fixed on the SPR chip, sterilized SARS-CoV (1:5 diluted with acetic acid buffer, pH 4.4) was pumped into the microfluidic device and the reaction between the antibodies and SARS-CoV was recorded in Fig.5. However, from the Fig. 5, there was no obvious light intensity increase of the reaction line, which may be caused by spatial obstacles which keep antibodies from contacting SARS-CoV.

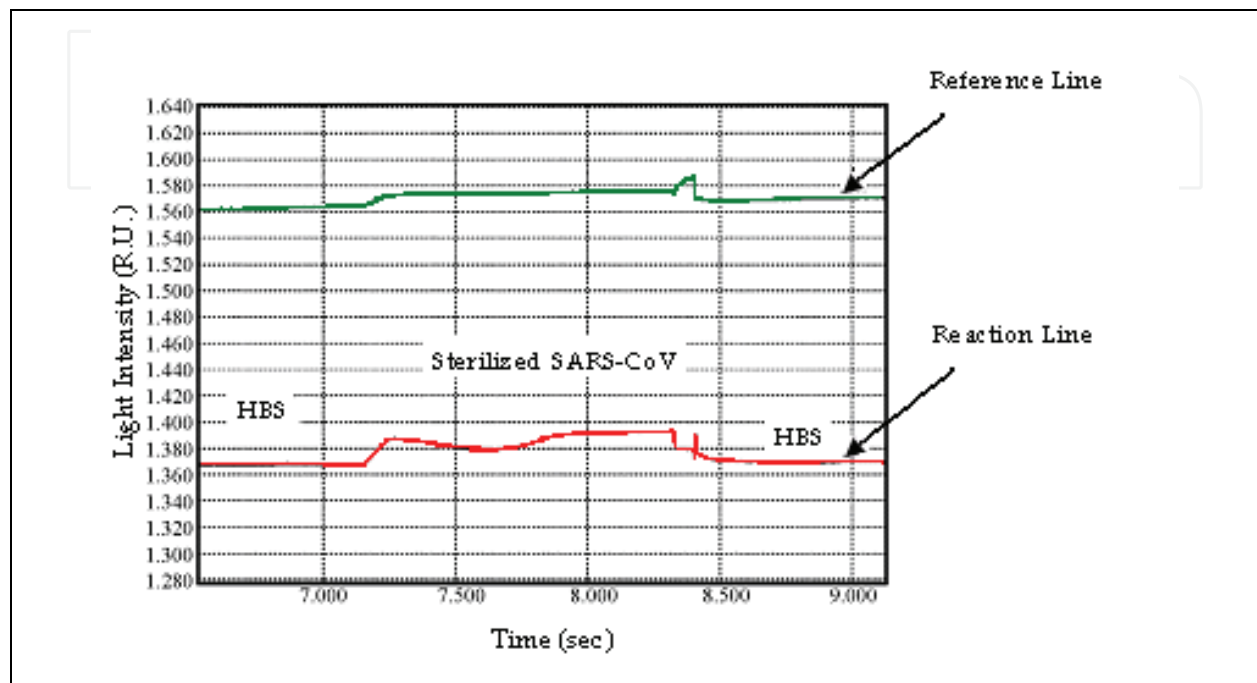


Fig. 5. Detection of sterilized SARS-CoV by immobilizing antibodies directly  
The green line is a reference curve. The red line is a reaction curve.

#### 4.3 Detection of SARS-CoV by using Protein A

In the type II design, 0.5mg/ml Protein A solution was pumped in after the activation step. The solution of the monoclonal antibodies to SARS-CoV (pH 4.6) was flushed in after the deactivation step, aiming to bind with immobilized protein A. Then the chip was washed with PBS buffer until the base-line signal was stable. After that, the same sterilized SARS-CoV solution (1:5 diluted with acetic acid buffer, pH 4.4) was pumped into the microfluidic device to react with immobilized antibody. The entire procedure was recorded, shown in Fig. 6, which demonstrates that the antibody to SARS-CoV was immobilized efficiently onto the reaction area of the SPR chip with the detection signal of SARS-Cov increasing to 60 units. It is 55 units higher than the reference signal with the binding rate about 1.4unit/min. Protein A has pseudoimmune interaction with the Fc fragments of immunoglobulins (Cedergreen et al, 1993) that could extend the distance between the antibodies and the surface so that the free degree of the antibodies could be increased. At the same time Fab fragments which react with antigens could face the solution side due to Protein A. Protein A seems to be a useful agent for antibody immobilization by increasing the binding efficiencies between antigens and antibodies.

Reference area was used to detect absorption of impurity in analyte, in order to eliminate the disturbance of non-specific absorption. The sensitivity of SARS-CoV detection reached  $1.66 \times 10^4$  PFU/mL.

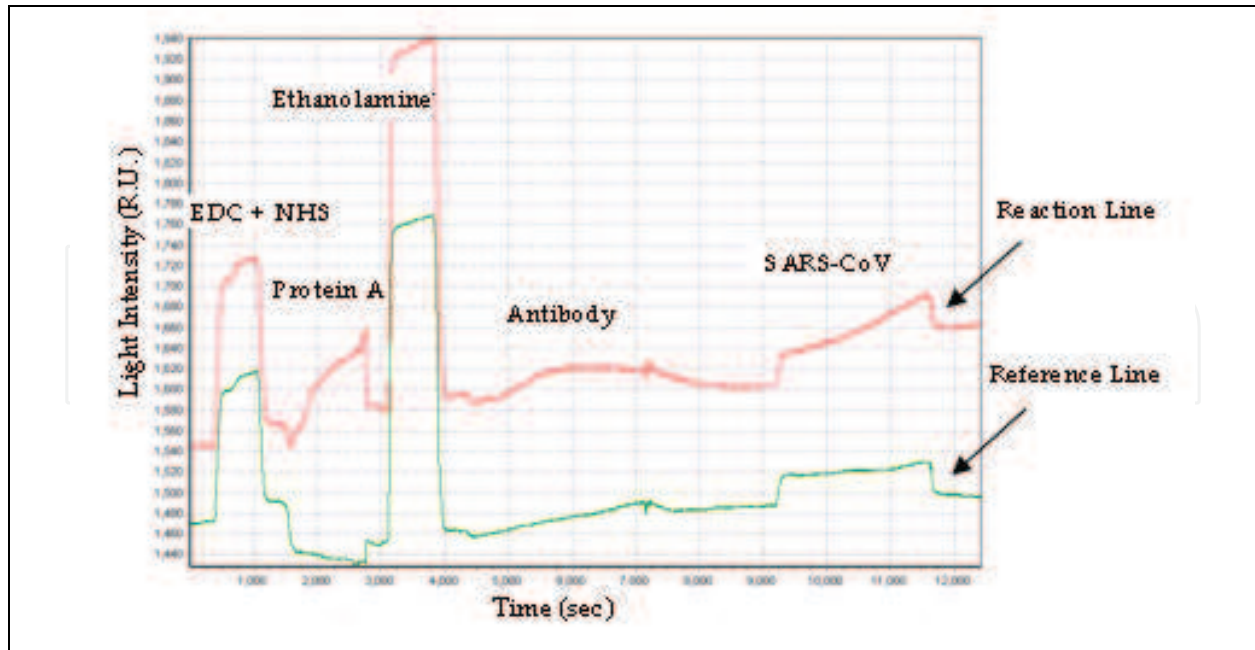


Fig. 6. Detection of sterilized SARS-CoV using Protein A  
The green line is a reference curve. The red line is a reaction curve.

#### 4.4 Detection of IgG by using an improved reference SPR system

This Reference SPR Analytical System used to detect SARS-CoV is an analytical instrument with one channel and two parameters in which only two analytes in one sample would be detected. The next generation of the reference SPR system: the high throughput, multi-analyte imaging SPR (HMI-SPR) analytical system, has been designed, fabricated and tested, shown in Fig. 7.



Fig. 7. Photograph of the high throughput, multi-analyte imaging SPR analytical system.

A 5-spots sensor chip was used as an example to briefly demonstrate the functions of HMI-SPR instrument. Firstly the 5-spots rare gold slides were modified by means of chemical methods. A thin layer of carboxymethyl dextran was immobilized on the gold spots of the slides to form a carboxyl-terminated surface. Then the mixed solution of EDC and NHS



(final concentrations are 0.2mol/L and 0.05mol/L) was used to activate the surface. 0.5 $\mu$ L solutions containing 1mg/mL rabbit IgG were dotted on the left two gold spots, while 0.5 $\mu$ L solutions containing 1mg/mL human IgG were dotted on the right two gold spots with the middle gold spot unmodified.

After the solutions were dotted on the gold areas, rabbit IgG and human IgG reacting with the dextran on the rare gold areas for 10min were fully immobilized on the chip. Then the sensor chip was deactivated by dipping in the ethanolamine solution of 1mol/L. During this process, the proteins which didn't bind to the sensor chip firmly were removed. After that, the sensor chip was put into the deionised water to wash away the remained salt solution. Finally, the modified sensor chip was mounted in the HMI-SPR system for different immunoassay tests.

The process of detection was conducted with the results of 5 real-time curves for 5-spots chip, shown in Fig. 8a. At first, HBS buffer was introduced for getting the base lines for 400s and then a solution containing 1.2mg/mL goat anti rabbit IgG was injected and stopped for reaction for 300s. Secondly, HBS buffer was introduced again for recovering the base lines for 400s and then a solution containing 0.5mg/mL goat anti human IgG was injected and stopped for reaction for 300s. Finally HBS buffer, HCl buffer and HBS buffer were introduced sequentially in which HCl buffer was used to bring the sensor chip back to the original setup. As shown in Fig. 8a, when goat anti rabbit IgG was injected, the immunoreaction occurred at rabbit IgG binding areas (curves 1 and 2). When goat anti human IgG was injected, the immunoreaction occurred at human IgG binding spots (curves 4 and 5). After HCl buffer was injected, all the gold areas of the sensor chip restored to base lines.

The linear calibration method of signal curves was performed to move base lines to zero and subtract the reference curve, shown in Fig. 8b.

Until now, we have designed, fabricated and tested 15-spot, 27-spot, 45-spot, 96-spot and 144-spot sensor chips.

## 5. Conclusion

In this chapter, a home-developed Reference SPR Analytical System has been demonstrated, in which the successful detection of the SARS-CoV antigens has been recorded by the measurement mode of fixed angle in which Protein A has been used to obtain a higher sensitivity. Then an improved reference SPR system, high throughput, multi-analyte imaging SPR (HMI-SPR) analytical system, was briefly illuminated by using a 5-spot sensor chip to detect two kinds of Immunoglobulin G. By using a reference area, the performance of the SPR system has been improved to avoid the refractivity inference of different solutions, different temperatures, and even to eliminate the disturbances due to non-specific absorptions. Therefore, the home-developed Reference SPR Analytical System is promising to implement clinic diagnosis by directly detecting the crude patients' samples without pretreatment procedures.

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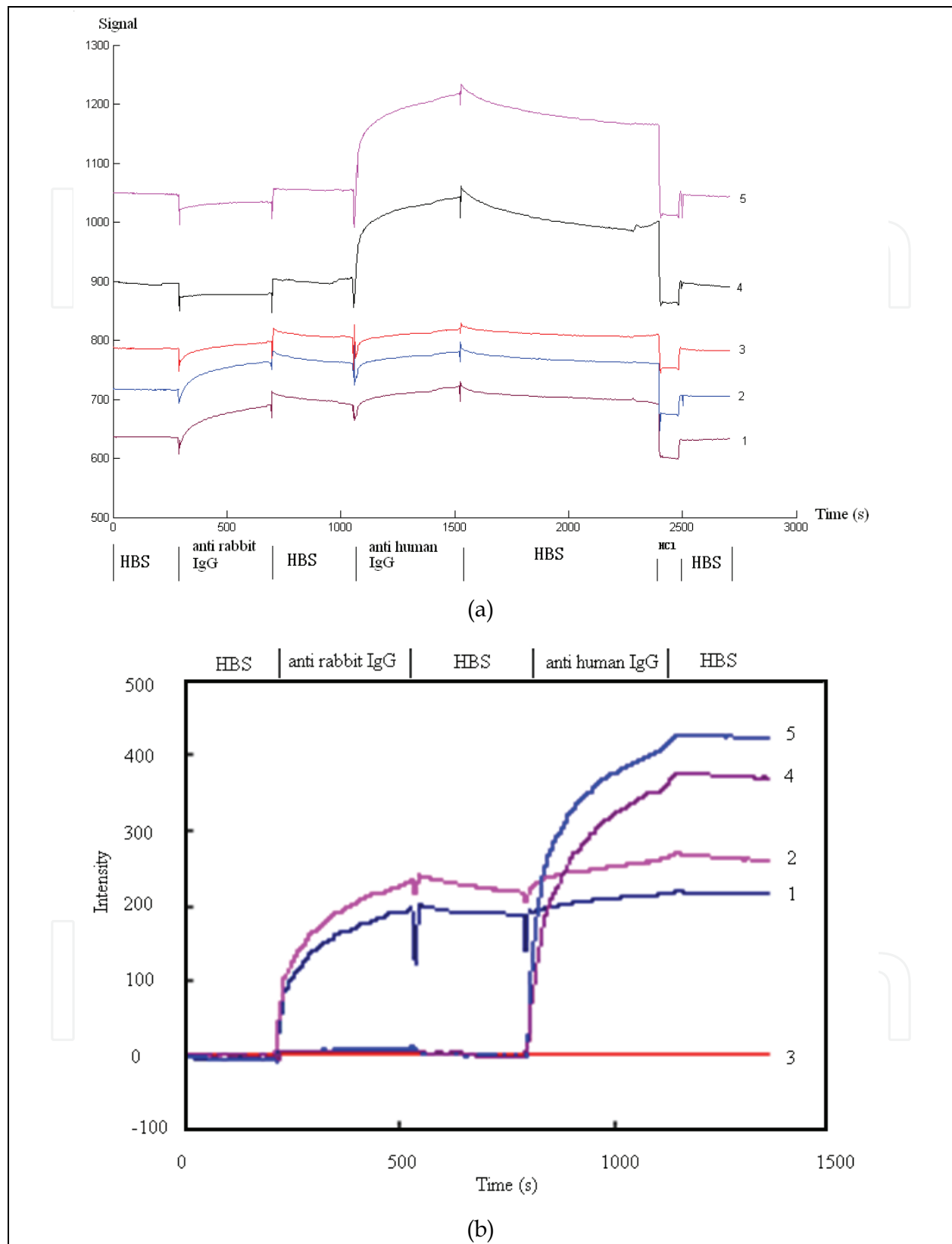


Fig. 8. SPR response curves for qualitative detections (a) before and (b) after data processed. Curves 1 and 2 for immobilization of rabbit IgG, Curves 4 and 5 for immobilization of human IgG, Curve 3 for reference.

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A biosensor is defined as a detecting device that combines a transducer with a biologically sensitive and selective component. When a specific target molecule interacts with the biological component, a signal is produced, at transducer level, proportional to the concentration of the substance. Therefore biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. Bringing together researchers from 11 different countries, this book covers a wide range of aspects and issues related to biosensor technology, such as biosensor applications in the fields of drug discovery, diagnostics and bacteria detection, optical biosensors, biotelemetry and algorithms applied to biosensing.

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