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Sensitivity Enhancement of Surface Plasmon Resonance Biosensor with Colloidal Gold

Kibong Choi[1]*, Heeju Youn[1], Kwangjoong Kim[1], and Jungdo Choi[2]

[1]GWSD-4-3, Agency for Defense Development, P.O. Box 35, Yusung, Taejon 305-600, Korea
[2]Department of Biochemistry, Chungbuk National University, Cheungju 361-763, Korea

We enhanced the sensitivity of surface plasmon resonance biosensor by the conversion of the real-time direct binding immunoassay into the sandwich immunoassay, in which colloidal gold particles coated with anti-mouse IgG was used. By the immobilization of anti-mouse IgG onto the carboxymethyl dextran surface of thin gold film, the direct binding of analyte (mouse IgG) onto the sensor chip, and the injection of colloidal gold particles coated with anti-mouse IgG, about 100 times of sensitivity enhancement was obtained. This result suggests that nanoparticles, which has a high refractive index, homogeneous ultrafine structure and capability of size control, would be applicable for the detection of very small quantity of biomaterial.

Key words: colloidal gold, surface plasmon resonance, sensitivity enhancement

INTRODUCTION

Nanobiotechnology is currently becoming an extensive growth area in application of biotechnology. Developing analytical/monitoring devices for investigation of biomolecular structure/behavior at molecular level and molecular-scale bioreactor for production of highly purified bioactive materials can be good example of nanobiotechnology [1-2].

Nanoparticle of gold has already been used in immunocytochemistry and colloidal gold-labeled immunoassay [3-4]. Colloidal gold particle was known to have high optical refractive index. Because of their ultrafine sizes and high surface area, these particle can easily overcome conventional restrictions of phase equilibria and kinetics, and can form active complexes with a wide variety of biological substances [5].

Real-time optical immunosensors, which is capable of direct observation of the interaction of macromolecules, were embodied in commercial reality [6]. Biosensors utilizing a biospecific binding reaction are often based on surface physical phenomena occurred when biomolecules interact on a solid surface.

Surface plasmon resonance (SPR) affinity sensors are based on the measurement of changes in the refractive index of the immediate vicinity of a metal surface. The reflectance becomes minimal at a certain angle, as shown in Fig. 1, when monochromatic polarized light in the plane of incidence is reflected by metal layer containing free-electrons such as gold, silver, copper and alumina. This minimum reflectance is sensitive to the changes in refractive index of the medium adjacent to the metal layer. Large molecules cause a considerable change in refractive index and a large shift in the plasmon resonance angle. BIAcore that was developed and introduced on the market by Pharmacia Biosensor is one type of optical immunosensors, and its detection principle is based on SPR. The reviews could be easily found in many scientific articles [7-8].

Despite of many advantages of SPR such as the real-time detection, the use of tag free reagents and the automated microfluidic system for treatment of small sample and reagents, low sensitivity has hindered the application of SPR in many area including micro-analysis and analysis of small molecules. As found in the trends of research and development of the other sensors, the avoidance of non-specific binding is also important [9].

Our effort was made to optimize and enhance the sensitivity of SPR using protein coated colloidal gold particles. The optimal conditions of immunochemical reaction for these complexes were investigated. And the comparison of sensitivities between direct immunoassay and sandwich immunoassay with colloidal gold particles in mouse IgG determination, was made.

MATERIALS AND METHODS

Reagents and Equipments

N-hydroxysuccinimide (NHS), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and ethanolamine were obtained from BIAcore AB Co. (Uppsala, Sweden). Mouse IgG, anti-mouse IgG, colloidal gold and all other reagents used were obtained from Sigma Chemical Co. (St. Louis, MO). BIAcore-X , as the sensor of SPR, and sensor chip CM-5 were obtained BIAcore AB Co. This device is equipped with an autoinjector, microfluidic system and software package for system control and data evaluation. Sensor chip CM-5 was modified with carboxymethyl dextran matrix on thin gold film of the chip.

Immobilization of Anti-mouse IgG on Sensor Chip

* Corresponding author
Tel: 042-821-4669 Fax: 042-821-2391
e-mail: bong525@chollian.net

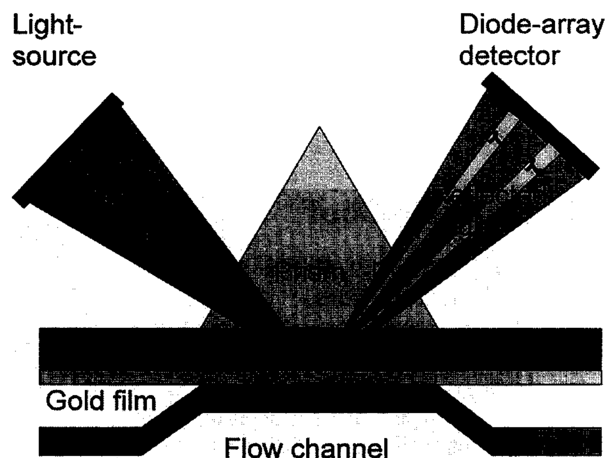

Fig. 1. The configuration of the surface plasmon resonance detector, sensor chip and flow channel in the BIAcore system. The detector works with polarized light from a light emitting diode, which is reflected in the gold film on the sensor chip and detected on a diode array. Surface plasmon resonance is observed as a decrease in light intensity for a specific angle of incidence. This angle changes with changes in the refractive index in the vicinity of the surface due to adsorption of large molecules on the immobilized ligand on the chip.

The same volume of NHS (11.5 mg/mL) and EDC (75 mg/mL) were mixed together, and then 35 μL of this mixture was injected into the chip for the activation of carboxymethylated dextran on surface of CM-5 chip. The flow rate and temperature were adjusted to 20 μL/min and 25°C, respectively, throughout the procedure of the activation and the immobilization. Immobilization of antibody on activated sensor chip was completed by the four successive injections of 35 μL of anti-mouse IgG (25 μg/mL) dissolved in 10 mM HEPES buffered saline (HBS, pH 7.4). Non immobilized part was deactivated using 35 μL of 1 M ethanolamine (pH 8.5).

Immobilization of Anti-mouse IgG on Colloidal Gold

The average diameter of colloidal gold was 20$\pm$2 nm, and concentration of colloidal gold solution was 4.6$\times 10^{11}$ particles/mL. Anti-mouse IgG (0.25 mg/mL, 15.2, 30.4 or 60.8 μL) dissolved in 25 mM carbonate/bicarbonate buffer (pH 9.0) was added to 1 mL of colloidal gold solution with gently stirring. After incubation for 2 hours at room temperature, 0.25 mL of 1% BSA solution in 25 mM carbonate/bicarbonate buffer (pH 9.0) was added to the above mixture and subsequently reacted for 2 hours at room temperature. The supernatant was removed by centrifugation at 15,000 rpm for 20 min at 4°C with microcentrifuge. The precipitation was washed with 10 mM phosphate buffered saline (PBS, pH 7.4), and the supernatant was removed by centrifugation. The precipitation was washed with PBS repeatedly, and was dissolved and diluted with PBS until O.D. reached 1.9 at 520 nm.

Assay of Mouse IgG Using SPR Sensor

To stabilize base line of SPR sensor, 10 mM PBS (pH 7.4) as a running buffer was injected at a rate of 20 μL/min into sensor chip immobilized with anti-mouse IgG. While 80 μL of mouse IgG (25 μg/mL) dissolved in 10 mM PBS (pH 7.4) was injected into sensor chip, the change in SPR angle was measured as a function of time. After washing with 10 mM PBS (pH 7.4), anti-mouse IgG coated colloidal gold was injected so as to attach colloidal gold onto the surface of sensor chip through the bridge formation of mouse IgG and anti-mouse IgG. After measurement, the regeneration of sensor chip was performed by the injection of 10 μL of 0.1 M HCl.

RESULTS AND DISCUSSION

Immobilization of Anti-mouse IgG on Sensor Chip and Colloidal Gold Particles

Coupling of anti-mouse IgG on sensor chip was carried out firstly, in order to determine how fast and strong this protein can be coupled to CM-5 chip through amine coupling and what is the optimum concentration of anti-mouse IgG.

The sensorgram in Fig. 2 shows the sequential procedures and responses for the immobilization of anti-mouse IgG to carboxymethyl dextran of CM-5 chip surface. The procedures include the activation of sensor chip with the mixture of NHS and EDS solution, four times' sequential immobilization of anti-mouse IgG (0.25 mg/mL) and deactivation of unreacted esters with ethanolamine solution. To maximize the quantity of immobilized anti-mouse IgG onto a given surface of sensor chip, sequential injections was performed. As the number of injection was increased, an immobilized quantity was increased but the rate of increase was diminished. When four times' injection was completed, the total increase of resonance unit (RU) was 12,913.4. According to the fact of that detection area of SPR is 0.224 mm^2 (1.4$\times$0.16 mm) and 1,000 RU corresponds to 1 ng/mm^2 of protein, total immobilized quantity of anti-mouse IgG onto SPR detection area was 2.89 ng (12,913.4 RU$\times$0.224 $mm^2\times$1 $ng/mm^2\cdot$ 1000 RU).

To examine the effect of colloidal gold coated with an-

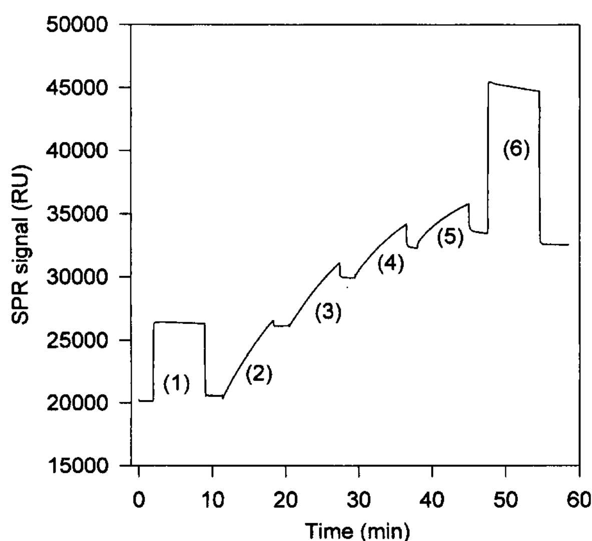

Fig. 2. The sensorgram of SPR sensor showing the sequential procedures and responses during the immobilization of anti-mouse IgG to carboxymethyl dextran on CM-5 chip. (1) Activation of carboxymethyl dextran with mixture of NHS and EDC, (2)~(5) sequential immobilization of anti-mouse IgG, and (6) deactivation of activated carboxymethyl dextran with ethanolamine.

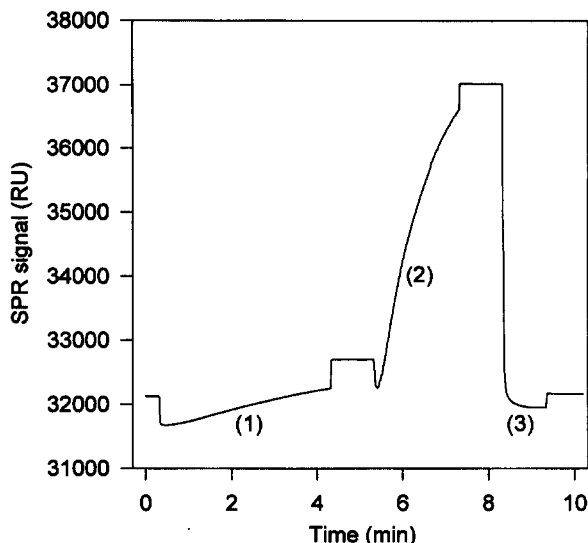

Fig. 3. The sensorgram of SPR sensor showing the synergistic enhancement of sensitivity using colloidal gold particles coated with anti-mouse IgG in sandwich assay format. (1) Binding of mouse IgG, (2) binding of colloidal gold particles coated with anti-mouse IgG, and (3) regeneration of sensor chip with HCl.

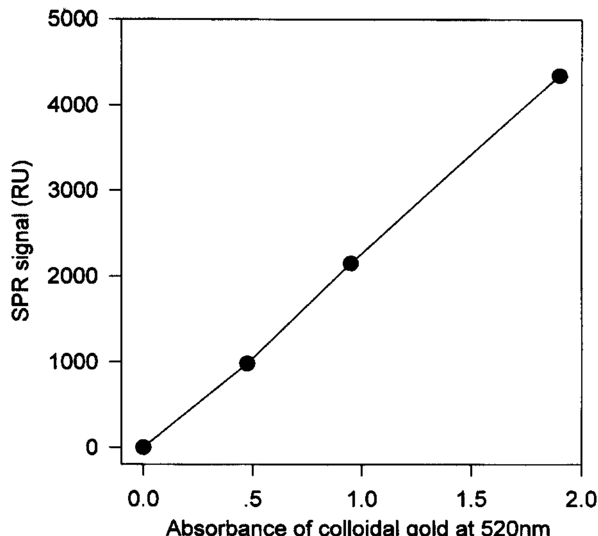

Fig. 4. Effect of the concentration of colloidal gold coated with anti-mouse IgG on SPR signal. Particles per A_{520} per mL is 5.1×10^{11}.

ti-mouse IgG on the signal amplification, we performed the preliminary experiments for direct immunoassay and sandwich immunoassay using colloidal gold particles in mouse IgG determination.

Mouse IgG solution (25 μg/mL, 80 μL) in PBS (pH 7.4, 10 mM) was injected to the anti-mouse IgG immobilized sensor chip, and then 40 μL of colloidal gold coated with anti-mouse IgG was injected. The initial ratio of anti-mouse IgG number to colloidal gold particle, in reaction solution for the complex conjugation, was adjusted to 100 : 1.

Sensorgram of Fig. 3 shows that sandwich immunoassay with colloidal gold coated with anti-mouse IgG have about 10 times higher RU signal than direct immunoassay.

It is desirable that the anti-mouse IgG is coated onto surface of colloidal gold particle to be in monolayer and perpendicular to the surface with its binding site faces outside. But this topological orientation can not be expected in practical experiment because a large number of anti-mouse IgG are immobilized on the surface of particle randomly by physical adsorption. To determine the maximum quantity of anti-mouse IgG which could be coated on a colloidal gold particle, the initial ratio of anti-mouse IgG to colloidal gold particle were adjusted to 50 : 1, 100 : 1 and 200 : 1, and signal amplications were compared. The highest signal was obtained when initial ratio of anti-mouse IgG to colloidal gold particle was 100 : 1.

The signal of 50 : 1 was about 40% of that of 100 : 1. There was little change in the signal of 200 : 1 compared with that of 100 : 1, and it was rather slightly reduced. These results suggest that the surface of gold particle was saturated with the anti-mouse IgG in the reaction condition, where the initial ratio of anti-mouse IgG to colloidal gold particles was 100 : 1.

Optimization of Sandwich Assay Based on the Anti-mouse IgG-gold Particles

To enhance sensitivity and to lower detection limit of mouse IgG sandwich assay with colloidal gold in SPR sensor, the optimum conditions for the concentration and the injection flow rate of colloidal gold coated with anti-mouse IgG, were investigated.

After addition of 80 μL of mouse IgG solution (25 μg/mL), 40 μL of collidal gold coated with anti-mouse IgG was injected. The optimum concentration of colloidal gold coated with anti-mouse IgG was determined by measuring absorbance at 520 nm. The number of 20 nm colloidal gold particles in 1 mL of colloidal gold solution where absorbance at 520 nm is 1.0, is known to be 5.1×10^{11} particles [10].

The absorbances at 520 nm for 2.43×10^{11}, 4.85×10^{11} and 9.7×10^{11} particles/mL colloidal gold solutions were 0.48, 0.95 and 1.9, respectively. The signal of SPR was linearly increased, proportional to the concentration of the gold particle as shown in Fig. 4. 9.7×10^{11}/mL solution had the highest SPR signal. More higher concentration of colloidal gold will show higher

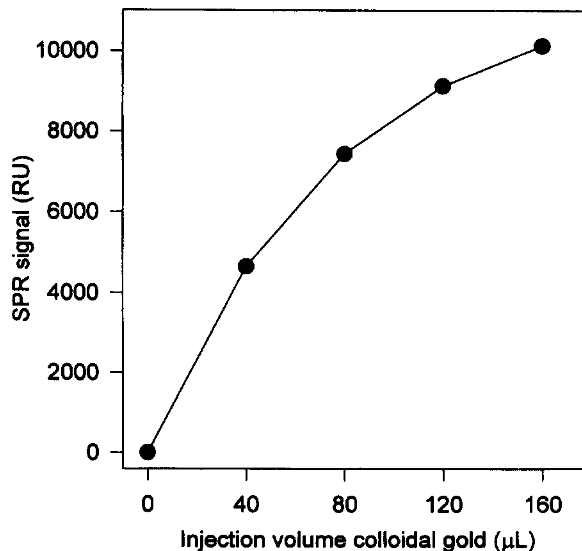

Fig. 5. Effect of the injection volume of colloidal gold coated with anti-mouse IgG.

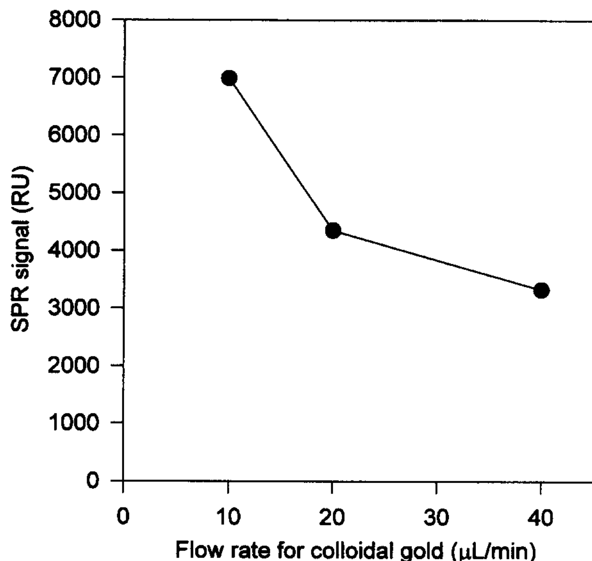

Fig. 6. Effect of the flow rate for colloidal gold coated with anti-mouse IgG.

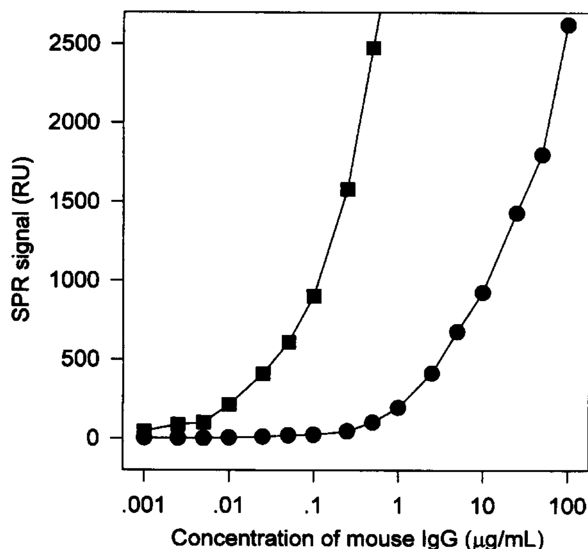

Fig. 7. Comparison of detection limit between direct binding assay and sandwich immunoassay using colloidal gold particles. Direct binding assay (●), sandwich immunoassay using colloidal gold particles (■).

SPR signal. But there are some problem in obtaining more concentrated colloidal gold particle due to their coaguration. Thus this solution was used in following experiments.

To determine the optimum injection volume for colloidal gold solution (9.7×10^{11} particles/mL), the signal changes with various volume of colloidal gold solution were measured as shown in Fig. 5. Signal was increased as the volume of particle solution was increased. The saturation phenomena was seen at the volume of 160 μL. The volume of particle solution was fixed as 80 μL in the following experiments to reduce reagent volume and reaction time. To determine the optimum flow rate for colloidal gold solution, signal change with three different flow rates (10, 20, and 40 μL/min) were measured as shown in Fig. 6. The flow rate of 10 μL/min showed the highest RU of 7,000, and that of 40 μL/min showed the lowest RU of 3,500. Flow rate is inversely proportional to reaction time. At high flow rate, there is no enough time for the binding of colloidal gold coated with anti-mouse IgG to mouse IgG on sensor chip.

Because the aim of this experiment is in the comparison of sensitivities between direct immunoassay and sandwich immunoassay with colloidal gold particles in mouse IgG determination, the flow rates for both assays should be the same. Thus, the flow rate of colloidal gold coated with anti-mouse IgG was adjusted to 20 μL/min to minimize reaction time for binding, although high SPR signal was observed at low flow rate.

Comparison of Direct Immunosasay and Sandwich Immunoassay with Colloidal Gold Particles

Based on the reaction condition which was optimized in the above section (concentration: 9.7×10^{11} particles/mL, volume of colloidal gold: 80 μL, injection flow rate: 20 μL/min) we compared the sensitivity of sandwich immunoassay with colloidal gold particles to the direct immunoassay of mouse IgG.

The SPR signals of direct immunoassay and sandwich immunoassay with colloidal gold particles were shown in Fig. 7. The curves for both assays exhibited similar pattern. But the curve for sandwich immunoassay with colloidal gold particles shifted to 100 times lower concentration of mouse IgG compared with direct immunoassay.

It is not obvious whether the sensitivity enhancement directly depends on the high refractive index of gold particle or not. However we could assume that almost entire surface of gold particle is expected to be coated with anti-mouse IgG. Although there is some residual bare surface, it may be covered with BSA by the following sequential treatment of BSA. So it could be thought that both high refractive index of gold particle and proteins on colloidal gold have actually influenced upon the sensitivity enhancement.

In previous study [11], the detection limit of mouse IgG with enzyme linked immunosorbent assay (ELISA) was 390 ng/mL.

The detection limit of direct immunoassay of mouse IgG with SPR was about 1 μg/mL which is larger than that of ELISA. As mentioned above, low sensitivity is one of defects of SPR. Thus SPR sensor has been thought to be inadequate in microassay. But we enhanced the sensitivity about one hundred times by the use of colloidal gold. The detection limit of sandwich immunoassay with colloidal gold was about 10 ng/mL which is about forty times smaller than that of ELISA. Our results indicates that colloidal gold technology can broaden the area of SPR sensor to microassay and assay of small molecules which has little effect on refractive index.

REFERENCES

[1] Laval, J. M., J. Chopineau, and D. Thomas (1995) Nanotechnology: R & D challenges and opportunities for application in biotechnology. *Trend in Biotech.* 13: 474-481.

[2] Davis, S. S. (1997) Biomedical applications of nanoyechnology-implications for drug targeting and gene therapy. *Trend in Biotech.* 15: 217-224.

[3] Birrell, G. B., K. K. Hedberg, and O. H. Griffith (1987) Pitfalls of Immunogold labeling: analysiss

by light microscopy, transmission electron microscopy, and photonelectron mircoscopy. *J. of Histochem. Cytochem.* 35: 843-853.

[4] Leung, P. T., D. P. Knight, G. P. Malan, and M. F. Finlan (1994) Modelling of particle-enhanced sensitivity of the surfase-plasmon-resonance biosensor. *Sensors and Actuators* B22: 175-180.

[5] Watts, H. J. and C. R. Lowe (1994) Optical biosensor for monitoring microbial cells. *Anal. Chem.* 66: 2465-2470.

[6] Liedberg, B., C. Nylander, and I. Lundstrom (1995) Biosensing with surface plasmon resonance-how it all started. *Biosensors & Bioelectronics* 10: i-ix.

[7] Jönsson U. and M. Malmqvist (1992) Real time biospecific interaction analysis; The integration of surface plasmon resonace detection, general biospecific interface chemistry and microfluidics into one analytical system. p.291-337. In A.P.H. Turner (ed.) *Advances in Biosensors, Vol. 2*, JAI Press, NY.

[8] Welford, K. (1991) Surface plasmon-polariton and their uses. *Optical and Quantum Electronics.* 23: 1-27.

[9] Karlsson, R. and A. Falt (1997) Experimental design for kinetic analysis of protein-protein interaction with surface plasmon resonance biosensors. *J. Immuno. Meth.* 200: 121-133.

[10] Ackerman, G. A. (1983) Differential surface labeling and internalization of glucagon by peripheral leukocytes. *J. Histochem. Cytochem.* 31: 433-440.

[11] Choi K-B., H-J. Youn, S-H. Cha, and J-D. Choi (1997) Measuring charateristics of mouse-IgG immunoassay with light addessable potentiometric sensor. *Kor. J. of Biotech. and Bioeng.* 12: 407-412.