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A protein detection technique by using surface plasmon resonance (SPR) with rolling circle amplification (RCA) and nanogold-modified tags

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Abstract

Surface plasmon resonance (SPR) can detect molecules bound to a surface by subtle changes in the SPR angle. By immobilizing probes onto the surface and passing analyte solution through the surface, changes in SPR angle indicate the binding between analyte and probes. Detection of analyte from solution can be achieved easily. By using rolling circle amplification (RCA) and nanogold-modified tags, the signals of analyte binding are greatly amplified, and the sensitivity of this technique is significantly improved. Furthermore, this technique has potentials for ultra-sensitive detection and microarray analysis. In this paper, this detection technique is introduced and shown to have great amplification capability. Using 5 nm nanogold with 30 min of RCA development time, this proposed protein detection technique shows over 60 times amplification of the original signal.

Keywords: Surface plasmon resonance; Protein array; Rolling circle amplification; Nanogold

1. Introduction

Visualization by fluorescence has become the standard method for detecting biomolecules such as protein and DNA. However, there are numerous examples where important biological markers for cancer, infectious disease, or biochemical processes have a concentration too low to be detected by conventional fluorescence detection techniques. One approach for improvement is to develop stronger fluorochromes and chemiluminescent substrates for ELISA, immunofluorescence-based staining, and immunoblotting (Chan et al., 2004; Seydack, 2005). Although these techniques can be quite powerful in some cases, greater sensitivity and specificity are often required when the sample material is limited or when the antigen density is low. Other directions of research include improving the sensitivity by amplifying the signal through DNA amplification (such as PCR); and developing new optical detection techniques that does not utilize fluorescence.

SPR is an optical detection technique based on reflection and refraction. In SPR, a solution containing the target molecules flows across a chip with probes immobilized on the surface. On the other side of the chip is a thin film of metal (usually gold). When plane-polarized light hits the gold film at a certain angle under total internal reflection conditions, light will be absorbed by the gold film and changed into surface plasmon on the gold surface (Bohren and Huffman, 1998). The mass of the molecules bound on the gold film varies proportionally to the SPR angle. Therefore, as the solution flows by, target molecules bind to the probes and change the SPR angle. Since the detection of target molecule only requires the solution to flow pass the chip, SPR has a significantly easier procedure than fluorescent techniques. The stability of the sample is increased because the light beam does not pass through the sample but only reflects from the sample. Without the bleaching problem of the fluorescence dyes, high intensity of light can be applied to shorten the detection time. Furthermore, the adsorption characteristics can be evaluated continuously with time. This allows kinetic, affinity, and mass transport measurements. Automation of the system enables accuracy and precision in all steps of the analysis, and increases both ease of use and

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reproducibility (Nelson and Krone, 1997; Rich and Myszka, 2001).

Application of nanogold (nanometer-scale gold particle) in SPR was shown to increase the SPR angle shift. Despite the fact that large colloidal particles are typically non-ideal as tags for real-time analysis due to slow diffusion kinetics, steric hindrance, and an increased opportunity for multivalent binding, they would be excellent as tags for standard detection of extremely low quantities of target molecules. As a result, detection sensitivity is improved when compared with other non-nanogold binding events. These results also demonstrate the potential for significant improvement in the sensitivity and dynamic range of nanogold amplified detection method by altering the size of the nanogold particles (Lyon et al., 1998, 1999; He et al., 2000).

DNA amplification utilizing immuno-PCR has already been used for the detection of antibody–antigen complexes (Sano et al., 1992; Ruzicka et al., 1993; Zhou et al., 1993). Furthermore, immuno-PCR using different DNA tags has been used to analyze multiple antibody–antigen complexes simultaneously (Hendrickson et al., 1995). Despite the increase in detection sensitivity, the needs for thermal cycling and product separations by gel electrophoresis have restricted the widespread adoption of immuno-PCR amplification technique. Isothermal rolling circle amplification (RCA) is a recently developed DNA amplification technique that does not require thermal cycling or product separation (Lizardi et al., 1998). In RCA, DNA polymerases are used to synthesize a DNA strand from a circular DNA template to amplify the signal. Using a single primer, RCA can generate a DNA strand consisted of hundreds of identical DNA sequences complimentary to the circular template within a short period of time (Fire and Xu, 1995). By attaching the 5′ end of the primer to an antibody, the antibody will be attached to a DNA strand consisting of multiple repeats of DNA sequence complimentary to the circular template. The repeated DNA sequence allows the antibody be detected by tags that contain the same DNA sequence as the circular template.

In this paper, a new biomolecules detection method utilizing both RCA and nanoparticle amplified SPR is introduced. By attaching nanogold to a DNA sequences which are similar to the one on the circular template, RCA product can be characterized by these nanogold-modified oligonucleotide tags (Hsu and Huang, 2004). For instance, using silver enhancement, detection of protein and DNA can be visualized by the nanogold-modified oligonucleotide tags. In this paper, SPR was used to detect these nanogold-modified oligonucleotide tags. The characteristics of SPR sensorgram, signal amplification capability and chip regeneration process were also studied.

2. Methods

2.1. Method of measurement

In order to modify the sensor chip surface with probes, a short fragment of SH-C_8-COOH linker was used to attach the probes to the surface. The choice of probes depends on the analyte. For example, if the analyte is a protein, antibodies that bind to this protein would be used as the probes. Once the sensor chip surface is modified with probes, the change of mass on the surface will produce a change in the SPR signal when the analyte solution flows across the surface and results in the binding between analyte and probes. From measuring the change in SPR signal, analyte from the solution can be detected.

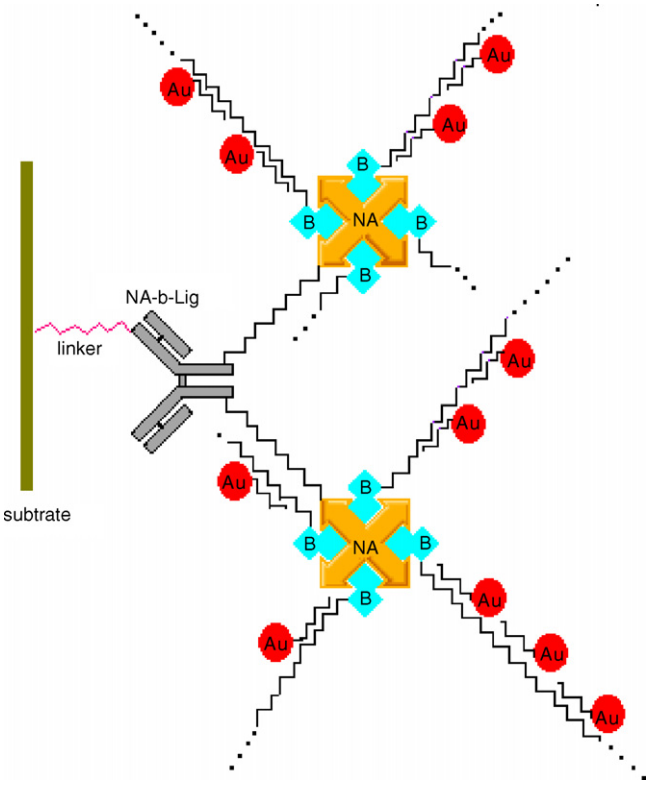

Fig. 1. Schematic illustration of the sensor chip surface after amplification.

The signal was then amplified by attaching more mass to the sensor chip surface, which would increase the change in the SPR signal. Rolling circle amplification (RCA) was performed on the second set of probes using the primer attached. With the addition of circular template, DNA polymerase, and nucleotides, RCA will replicate a strand of DNA from the primer. With increased mass provided by the replicated DNA strand, the change in SPR signal is amplified. Since nanogold induce a significant change in the SPR signal, by modifying the oligonucleotides tags with nanogold and adding to tags to hybridize with the replicated DNA strand, the change in SPR signal is amplified even further. The schematic illustration of the sensor chip surface after amplification is shown in Fig. 1.

2.2. Preparation of nanogold particles

Nanogold particles were prepared using a method of citrate reduction of $HAuCl_4$. All glassware were cleaned in aqua regia to wash away any possible gold residue. First, reflux 50 mL of 0.01% aqueous solution of $HAuCl_4$ while stirring. Then, quickly add 1% trisodium citrate solution into the refluxing $HAuCl_4$ solution, which resulted in a change of color. Different amounts of trisodium citrate added will result in different sizes of

Table 1
Oligonucleotide sequence designs

Oligonucleotides ID	Sequences	Description
Primer	5′-AAA AAA AAA AAA TAA TAC GAC TCA CTA TAG GGA ATT CCG-3′	5′ end modified with a biotin group Interacts with avidin-related protein
Circle template	5′-CCC TAT ACA CCC AAG CTT CCC ACC GGA ATT-3′	5′ end is phosphorylated for further ligation reactions to become circular
Complementary-1	5′-CGG AAT TCC CTA TAG TGA GTC-3′	Sequences directly complementary to primers 5′ end modified with a SH group, for reacting with gold particle surface
Complementary-2	5′-CAC CCA AGC TTC CCA C-3′	Sequences complementary to putative RCA concatamer products 5′ end modified with a SH group, for reacting with gold particle surface

nanogold particles, ranging from 15 to 150 nm in radius (Hsu and Huang, 2004). For the synthesis of 5 nm nanoparticles, the protocol was slightly modified. 225 μL of 1% tannic acid was mixed with 1 mL of 1% trisodium citrate, and then added to the refluxing $HAuCl_4$ solution. After the color changed, the solution was refluxed for an additional 15 min, allowed to cool to room temperature, filtered through a 0.22 μm filter, went through dialysis, then stored at 4 °C. The nanogold particles were characterized by TEM. Average particle diameter and standard deviations were determined by particle size analysis of TEM images using NIH Image J 1.29 software (a public domain program, free download at http://rsb.info.nih.gov/ij/).

2.3. Preparation of oligonucleotides used for RCA

The oligonucleotide sequences were designed and listed in Table 1. The oligonucleotides used were produced by MDBio, Inc. (Taiwan). The primer sequence was designed, and the 5′ ends were modified with biotin to attach to the avadin on the antibody. The circle template has one segment of sequences that is complimentary to the primer sequence for primer binding, and a second segment of sequences designed for use in the binding of oligonucleotide tags. Two types of complementary sequences were designed for use as oligonucleotide tags to bind to the replicated DNA strand produced from the circle template. Complementary-1 is complimentary to primer sequence, and Complementary-2 is the same as the second segment on the circle template.

2.4. Preparation of nanogold-modified oligonucleotide tags

First, mixed 5 mL of the desired aqueous nanogold solution with complementary oligonucleotides (3.1 μM). Allowed the solution to stand for 16 h, and then brought the solution into 0.1 M NaCl and 10 mM phosphate buffer (pH 7). Allowed the solution to stand for further 40 h, and followed by centrifugation for at least 25 min at 14,000 rpm to remove excess reagents. Removed the supernatant, and washed the red oily precipitate with 5 mL of a stock 0.1 M NaCl, 10 mM phosphate buffer (pH 7) solution, recentrifuged, and suspended in 5 mL of a 0.3 M NaCl, 10 mM phosphate buffer (pH 7), 0.01% azide solution.

2.5. Preparation of primer–template complexes

Primer–template complexes were prepared using a method of dilute annealing followed by ligation. First, 1.2 nmol of circular template oligonucleotides and 1 nmol of primer oligonucleotide were mixed with 280 μL of ligation buffer (50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/mL BSA), and incubated at 23 °C for 30 min. Then 120 Weiss units of T4 DNA, ligase purchased from NEB, was added and stored at 16 °C for 4 h. After storage, ligation was stopped by the addition of stopping buffer (10 mM EDTA, 0.1% SDS, 1 M NH_4OAc, 50 μg glycogen). Primer–template complexes were extracted with 1:1 phenol/chloroform, and then with chloroform. Then primer–template complexes were precipitated out with ethanol. Final products were resuspended in 60 μL of double distilled H_2O and stored at −20 °C. The whole process was performed under low temperature to avoid denaturation.

2.6. Setting up SPR apparatus

SPR measurements were taken using a Biacore J instrument (Uppsala, Sweden). The method for performing the analysis was to inject analyte solution over a modified surface of a gold film sensor chip from the instrument. To calibrate the instrument, standard refractive index solutions were used to obtain a calibration scale factor (angle shift/refractive index shift) in units of °/RU. First, 70 μL of 5 mM SH-C_8-COOH linker fragments were injected across gold film sensor surfaces in the Biacore apparatus at 37 °C with a flow rate of 10 μL/min in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.0005% Tween 20. Then 35 μL 50 mM NHS/200 mM EDC solution was injected at a flow rate of 5 μL/min to activate the surface for further immobilization of probes such as oligonucleotides or proteins. Next, 35 μL of 1 M ethanolamine (pH 8.5) was injected for deactivating excess reactive groups. When the desired degree of immobilization was achieved, the surface was washed by a 10 μL pulse of 0.5% SDS at a flow rate of 20 μl/min.

2.7. RCA development in SPR apparatus

In the RCA development process, approximately 5 units of DNA polymerase and 1 mM of dNTP were injected at a flow rate of 2 μL/min across surfaces in the BIAcore apparatus at 37 °C in a buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20. Three different time intervals of RCA development period of 0, 30, and 50 min were tested. Nanogold-modified oligonucleotide tags were injected at 10 μL/min for amplifying the signals. Three different nanogold diameters of 5, 16, and 30 nm were

tested. Interactions are time-dependent, and so SPR data were recorded continuously in the sensorgram.

2.8. Regeneration of SPR apparatus

Upon the completion of the procedure, solutions were added for the dissociation of the nanogold-modified oligonucleotide tags and the regeneration of the probes on the sensor chip surface. For this process, it required at least three times of injecting a series of regeneration buffers (35 μL pulses at 20 μL/min): 10 mM acetate (pH 4.8), then 10 mM glycine (pH 3), and finally 10 mM glycine (pH 2).

2.9. Atomic force microscopy (AFM)

Before the regeneration of SPR apparatus, the surface morphology of the surface of the gold film sensor chip (schematically shown in Fig. 1) was characterized using an atomic force microscope (AFM, SMENA Scanning Probe Microscope, NT-MDT Co., Russia). A standard etched silicon probe (SC series of Ultrasharp Silicone Cantilever, NT-MDT, Russia) was used for the AFM.

3. Results and discussion

3.1. SPR detector calibration

Since the SPR technique has a very high sensitivity for changes in refractive index of the solution, the practical unit used for measuring SPR signals is defined as 1 RU = 10^{-6} RIU (refractive index unit). From calibration of this experimental setup, a change of 1 RU in the refractive index of the solution caused a SPR angle shift of approximately 0.0001°. Note that this value will differ between different experimental setups, because the SPR angle shift depends not only on the refractive index of the solution, but also the choice of supporting metal, the metal coating, substrate glass, and wavelength of incident light. The effect of temperature drift at the sensor chip surface is less than 0.3 RU/min, which is negligible when compared with the amplitude of typical SPR signals. This ensures precision of the measurements.

3.2. SPR measurement

The SPR signals is recorded in a sensorgram, which records the SPR signals in units of RU over a period of time. A typical SPR sensorgram for the whole SPR procedure is shown in Fig. 2. The sensorgram showed that as sample solution passed through the sensor chip, SPR signal either increased or decreased. After the sample solution had passed, the SPR signal returned to a value closer to the one before the sample injection. The difference between the SPR signals before and after the sample injection is due to the amount of molecules on the sensor chip surface. This showed that as the sample solution passed through the sensor chip, molecules were exchanged between the solution and the sensor chip surface. Binding or dissociation of molecules on the sensor chip surface was controlled by injecting different solutions.

Since the SPR signals are affected by the amount and the type of molecules attached to the sensor chip, all SPR signals should be compared to a reference value. Normally, the reference value is the SPR signal after the probes are fixed onto the sensor chip, but before the addition of the analyte solution. After the addition of the analyte solution, the relative SPR signals, which are the SPR signals minus the reference value, represent the amount of analyte attached to sensor chip. In this experiment, the reference value is when the sensor chip surface is clean without anything is added. From Fig. 2, we can obtain the reference value is around 17,300 RU (first plateau, before anything is added). After the injection of 5 mM linker of SH-C_8-COOH, the SPR signal is leveled again at around 17,750 RU. Subtracting the reference value from it gives approximately 450 RU; it is the relative SPR signal of the linker attached to the sensor chip.

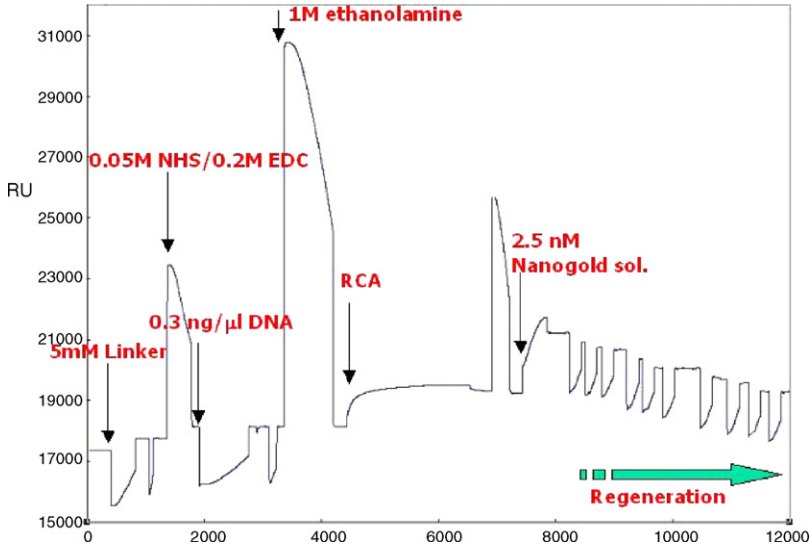

Fig. 2. A SPR sensorgram of a typical SPR procedure.

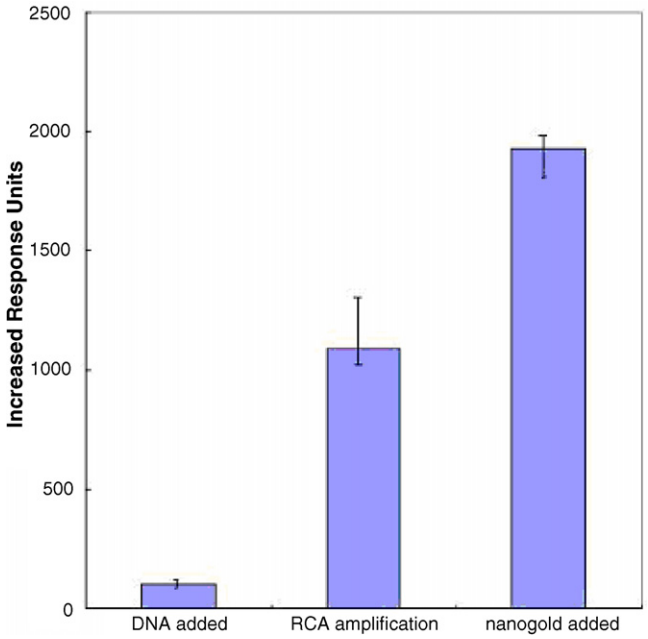

Fig. 3. Signal amplification by RCA process and nanoAu probes: ss-DNA as the targets.

Fig. 3 was the net signal increases on the studies with single strand-DNA (ss-DNA) as the targets. It showed that a typical SPR procedure using 16 nm nanogold-modified tags and 35 min of RCA development time amplifies the relative SPR signal significantly. After RCA but before the addition of nanogold-modified tags, the relative SPR signal had already achieved a magnification of 10× compared to the DNA-only. The signal amplification resulted from the increased mass on the sensor chip surface when a replicated strand of DNA was synthesized from RCA. With the addition of the nanogold-modified tags, the SPR signals increased even further, which obtained another magnification of about 2× compared with the previous step. The further amplification results could also be accounted by the increased mass from the nanogold-modified tags, which had been attached to the sensor chip surface.

3.3. Signal amplification using different nanogold diameters and RCA development times

In these experiments, combinations of three different RCA development time of 0, 30, and 50 min with three different nanogold diameters of 5, 16, and 30 nm were tested for the effects on signal amplification. The relative SPR signals results are shown in Fig. 4. Since the length replicated DNA strand generated from RCA is proportional to the RCA development time, one would expect a larger signal from longer RCA development time before the addition of nanogold-modified tags. The results showed that the relative SPR signals before the addition of nanogold-modified tags increased with longer RCA development time.

Since the relative SPR signals are proportional to the mass of molecules bound on the sensor chip surface, one would expect that the 30 nm nanogold to have the largest signal because of its larger mass. Interestingly, 5 nm nanogold-modified tags generated the best amplification result when compared with the other two diameter sizes. This could be attributed to the smaller steric effects from the 5 nm nanogold, since the replicated DNA strand is only a few hundred nm long. Because of its smaller size, more tags are able to hybridize onto the same length of replicated DNA strand and produce a larger relative SPR signal.

Since longer RCA development time replicates a longer DNA strand which fits more tags, one would expect an increase in the relative SPR signals with increased RCA development time after the addition of nanogold-modified tags. However, the results showed that for the 5 and 16 nm nanogold, 30 min RCA development time produced the best amplification effects, and 50 min of development time actually decreased the amplification effects. The main reason may be due to the incomplete regeneration of the surface. Since there was still certain level of nanoprobes not washed out, the binding sites though increased during the RCA reaction, however, the addition of nanoprobes for detection was not able to fully and accurately interpret the total amount of responses, which could actually contribute. It might be useful to do the correction that was to take the part of bounded probes into account and accumulate, but some errors would inevitably occur. Another unexpectedly phenomenon was the unequal amount

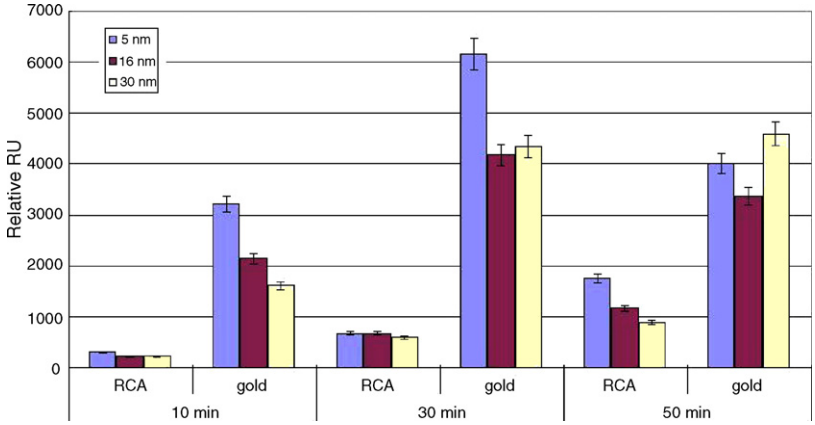

Fig. 4. Signal amplification by RCA process and nanoAu probes: antibodies as the targets (signal amplification using different nanogold diameters and RCA development times were compared).

of signals generated by RCA at 50 min since the same condition was actually applied in all three cases. Especially in the case of 30 nm nanoprobes, the efficiency of enzyme replication seemed decrease dramatically. The greater steric effect of 30 nm nanoprobes was proposed and this factor probably in someway affected the activity and efficiency of the polymerase to amplify the templates.

4. Conclusion

The proposed protein detection technique using SPR with RCA and nanogold-modified tags had shown the significant amplification of the original signal. Furthermore, the results indicated that using 5 nm nanogold with 30 min of RCA development time produced the greatest amplification effects. Future direction of research includes applying this technique to different analytes, optimizing this technique for best amplification effects, and miniaturizing the apparatus for use in microarray.

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