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Double-Enhancement Strategy: A Practical Approach to a Femto-Molar Level Detection of Prostate Specific Antigen-α_1-Antichymotrypsin (PSA/ACT Complex) for SPR Immunosensing

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Abstract Prostate specific antigen-α_1-antichymotrypsin was detected by a double-enhancement strategy involving the exploitation of both colloidal gold nanoparticles (AuNPs) and precipitation of an insoluble product formed by HRP-biocatalyzed oxidation. The AuNPs were synthesized and conjugated with horse-radish peroxidase-PSA polyclonal antibody by physisorption. Using the protein-colloid for SPR-based detection of the PSA/ACT complex showed their enhancement as being consistent with other previous studies with regard to AuNPs enhancement, while the enzyme precipitation using DAB substrate was applied for the first time and greatly amplified the signal. The limit of detection was found at as low as 0.027 ng/ml of the PSA/ACT complex (or 300 fM), which is much higher than that of previous reports. This study indicates another way to enhance SPR measurement, and it is generally applicable to other SPR-based immunoassays.

Keywords: Enzyme precipitation, gold nanoparticles, prostate specific antigen-α_1-antichymotrypsin complex, surface plasmon resonance, double enhancement, oligo (ethylene glycol)

Prostate carcinoma is a major cause of death in the male population. It is the most common tumor in men, the second leading cause of cancer deaths among males, and the fourth most common cause of death overall in the U.S. [2, 6]. The disease is increasing rapidly, which has led to the prediction that prostate cancer will become the most common cause of cancer causing death in men by the year 2010 [23]. At present, no curative therapy is available once the disease metastasizes to other sites in the body.

Prostate specific antigen (PSA), a 34-kDa serine protease, is the most reliable and specific biomarker for the preoperative diagnosis, monitoring, and follow-up of patients with prostate cancer [9]. It offers the best hope about an early and accurate detection while the disease is still localized in the prostate gland [5]. The major forms of PSA found in serum are complexes with two major extracellular serine protease inhibitors, α_1-antichymotrypsin (PSA/ACT, MW 90 kDa) and α_2-macroglobulin (PSA/AMG), and a free form (f-PSA, MW 34 kDa) [18]. PSA/ACT is the predominant form of the PSA complex; it is immunoreactive, whereas PSA/AMG is not. Conventional assays for PSA detection mostly involve a monoclonal or a polyclonal antibody of PSA tagged with an enzyme, a fluorophore, or a radioactive isotope [2, 16, 17]. These approaches had several disadvantages, such as being time consuming, inconvenient, and expensive. Furthermore, the immune reactions could not be observed in real time.

SPR, an affinity optical sensor based on the detection of changes in mass concentration at a biospecific interface [3, 12, 15, 21], has been applied to detection of PSA [4, 7, 13]. However, it is a fact that lack of a highly sensitive analytical method for detecting an analyte at low concentrations is a major impediment to SPR biosensor technology. To improve the sensitivity of PSA detection using SPR, AuNPs have been used because of their high mass as well as the asset of their ability to undergo surface plasmon resonance phenomenon leading to strengthening the SPR signal [11, 19, 20]. Recently, another strategy has noticeably emerged as a novel technique to amplify the SPR sensitivity, exploiting an HRP-catalyzed precipitation mechanism [8]. Alfonta *et al.* [1] prepared liposomes labeled with biotin and HRP to amplify antigen-antibody or oligonucleotide-DNA biosensing processes by the precipitation of an insoluble product on electrode. Su *et al.* [24] reported that the HRP-catalyzed oxidation of 4-CN could be applied to enhance the signals of cuvette-based surface plasmon resonance and quartz crystal microbalance (QCM) biosensors

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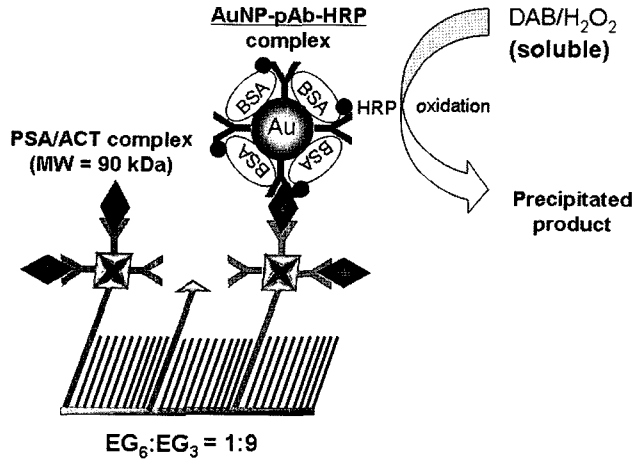

Fig. 1. Schematic diagram illustrating the steps in PSA/ACT complex detection and enhancement strategy based on the SPR immunosensor.

After the biotinylated PSA/ACT mAb was immobilized onto the SA layer, the PSA/ACT complex in HBS buffer was flowed over the 1:9 surface. Response of the antigen-antibody reaction was measured in real time using the BIAcore 2000 apparatus. Subsequently, HRP-PSA pAb-AuNPs were injected over the sensor surface to achieve better sensitivity.

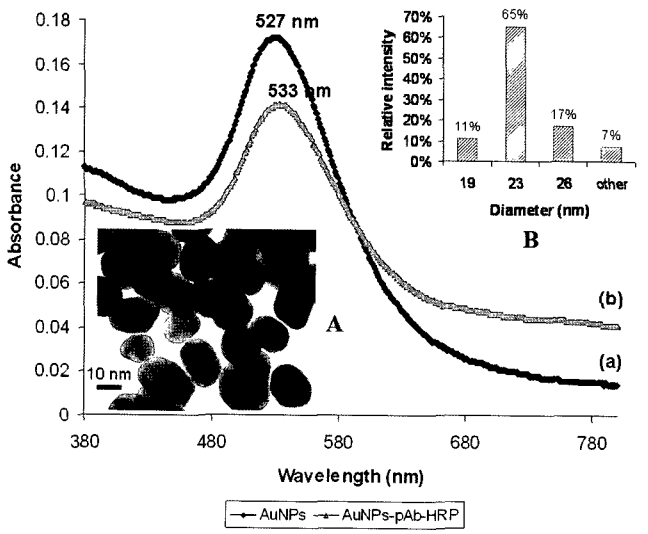

Fig. 2. UV-vis absorption spectra of (**A**) AuNPs; (**B**) Protein-gold conjugate.

Maximum absorptions of (**A**) and (**B**) are 527 and 533 nm, respectively. The inset A is the morphology of AuNPs characterized by HR-TEM using a JEOL model JEM-2100F instrument operated at an accelerating voltage of 200 kV. The inset B describes the size distribution of AuNPs measured by dynamic light scattering (DLS) analysis using a BI-200SM goniometer system and a BI-9000AT digital multiple correlator (Brookhaven Instruments, U.S.A.).

as well. In this note, we present a novel method, so-called double enhancement, for the specific PSA/ACT complex sensing process based on the application of HRP-labeled polyclonal antibody-functionalized gold nanoparticles. By functionalizing the AuNPs with HRP, we can exploit both the outstanding characteristics of gold colloid and enzyme precipitation sequentially for signal enhancement of the SPR biosensor. The strategy is illustrated in Fig. 1.

In the strategy, the first step involved preparation of colloidal gold nanoparticles. AuNPs were prepared by sodium citrate reduction of aqueous $HAuCl_4$ solution as described by Turkevich *et al.* [25]. A volume of 20 ml of 1.0 mM $HAuCl_4$ was heated to the boil, and 2 ml of 1% sodium citrate was added to the boiling solution while stirring vigorously. The solution was further boiled for 5 min to complete the citrate reduction of the gold ions. Then, the solution was kept on stirring for the next 30 min and cooled to room temperature. The formation of the monodisperse colloidal gold particles was indicated by a color change from dark blue to red. The colloidal solution was cooled and filtered through a 0.45-μm filter. Before use, the pH was adjusted to 7.2 by adding 0.5 N NaOH, and the solution was stored at 4°C. This method yielded spherical, quite homogenous, ruby red particles, as revealed by TEM (inset A, Fig. 2), and the size distribution was very narrow (D_{mean}=22.7 nm; RSD=3.51 nm) as measured by DLS (inset B, Fig. 2). Curve (a) in Fig. 2 shows the UV-vis spectrum of the ruby red solution produced after chemical reduction with sodium citrate, indicating formation of AuNPs with a maximum absorption peak at λ=527 nm, assigned to the characteristic feature of gold plasmon resonance [24].

PSA pAb was conjugated to HRP using the EZ-Link maleimide activated horse-radish peroxidase kit according to the manufacturer's protocol. Thereupon, the protein-colloidal gold nanoparticles were prepared by physisorption. Adsorption isotherms were constructed for the HRP-PSA pAb conjugate to determine the minimum amount of protein that was necessary to coat the exterior of the AuNPs [10, 14]. A series of protein solutions of increasing concentration was made, and 1 ml of colloidal AuNPs was added to 0.1 ml of the protein solution. After 5 min, 1 ml of 10% NaCl was added and rapidly mixed. If the protein was not adsorbed and the gold particles were not stabilized, aggregation of the gold granules was indicated by a color change from red to light blue. The minimum amount of protein that prevented this color change was used. For the *ca.* 23 nm colloid, 200 μg of HRP-PSA pAb/1 ml of colloid was required to prevent aggregation. The protein-Au conjugate was synthesized by the addition of the identified amount of HRP-PSA pAb to 1 ml of pH adjusted colloid followed by incubation on ice with periodic gentle mixing for 30 min. Then, 0.5 ml of freshly made and prefiltered (0.45 μm Millipore) containing 1% BSA and 0.1% Tween 20 were added to prevent aggregation of protein-coated gold particles. The protein-labeled gold particles were centrifuged at 12,000 rpm for 30 min. The clear to pink supernatant was discarded and the concentrated labeled gold pellet was resuspended in 2 ml of 0.1 M PBS buffer (pH 7.2). The enzyme immunogold solution was

stored at 4°C for up to 1 week without loss of activity. As a result, a damping of surface plasmon band occurred as indicated by a small red shift from 527 to 533 nm (curve (b), Fig. 2), and it showed the adsorption of HRP-PSA pAb onto the surface of AuNPs.

For real-time detection of the PSA/ACT complex, a BIAcore 2000 apparatus (Pharmacia Biosensor AB, Uppsala, Sweden) was used. The instrument was operated using the BIAcore 2000 control software; data were evaluated using BIAevaluation 3.2. In this study, to reduce any nonspecific adsorption and steric hindrance effect of protein immobilization as well as to maximize the capture protein quantity, a surface chip using a 1:9 molar ratio of HS-OEG_6-COOH/HS-OEG_3-OH was used [22]. The chip fabrication, biotinylation process, and streptavidin immobilization were carefully described in our previous publication [7]. Then, 20 μg/ml biotinylated PSA/ACT mAb was immobilized by injecting 35 μl over the flow cell 2 for 7 min at 5 μl/min. Flow cell 1 was considered as a reference cell for correction of the signal responses. The unbound biotinylated PSA/ACT mAb was washed away using a mixed solution of 25 mM NaOH/0.2 M NaCl for 2 min. To detect the analyte, the PSA/ACT complex was diluted with HBS buffer to yield concentrations ranging from 0 to 1,000 ng/ml. The analyte concentrations were injected over the immobilized surface for 5 min. After measuring the immune response, the sensor chip was regenerated by injecting 50 mM NaOH/1 M NaCl for 2 min. As shown in Fig. 3, the analyte could not be determined at low concentrations ranging from 0.001 to 1 ng/ml, and the linear range was only observed for concentrations ranging from 1 to 1,000 ng/ml (Fig. 4). A linear regression equation for the primary response was calculated as $y=0.1029x+9.8785$ ($R^2=0.9968$, n=5), where y and x are the relative RU and analyte concentration,

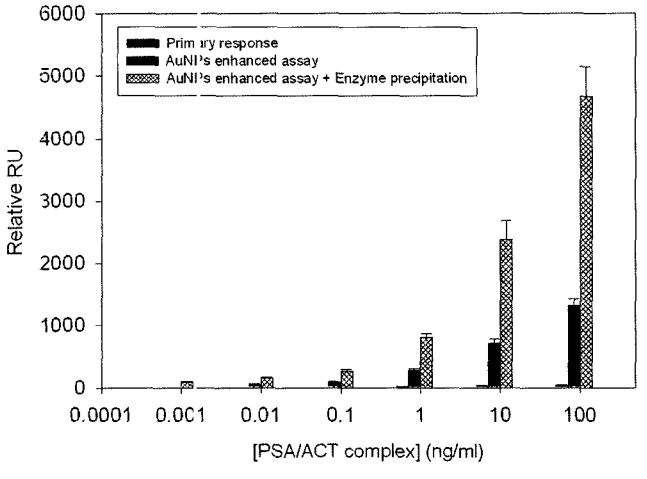

Fig. 3. Detection of the PSA/ACT complex at concentrations ranging from 0.001 to 100 ng/ml.
The error bars illustrate the relative standard deviation (RSD) for three replicates.

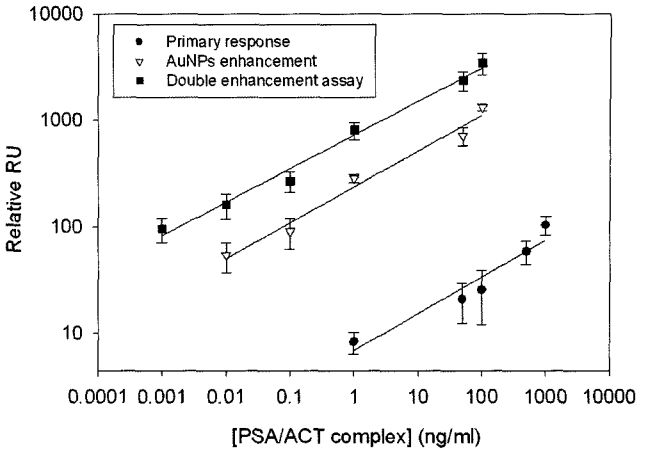

Fig. 4. Logarithmic correlation between the relative RU and the PSA/ACT complex concentrations.

respectively. The RSD of the zero concentration was 1.95. As defined, the limit of detection of the PSA/ACT complex is three times the standard deviation for the average measurement of blank samples [7]. Therefore, the LOD was determined to be as low as 56.9 ng/ml.

For the enhancement assays, the SPR signal was amplified sequentially in two steps as depicted in Fig. 1. After the immune response between the biotinylated PSA/ACT mAb and PSA/ACT complex had occurred, the HRP-PSA pAb-Au complex was injected over the surface for 5 min at 5 μl/min for the gold enhancement. Subsequently, a 0.2× DAB/H_2O_2 substrate solution was freshly prepared and injected to react for 2 min. The deposition of the oxidized insoluble product on the chip interface leads to SPR angle shifts and thus the sensitivity of SPR biosensor could be improved. Regeneration of the surface was carried out as above, or using glycine-HCl, pH 2.0, with a 2-min pulse. Interestingly, the relative RU values of the SPR immunoassays could be considerably improved by the enhancement strategy, as revealed in Fig. 3. The logarithmic correlation between the relative RU and the PSA/ACT complex concentration also exhibited a good linear relationship for the AuNPs enhancement and the double enhancement assay (Fig. 4). Linear regression equations were also obtained by the same calculation as the previous section. In the AuNPs enhanced assay, the analyte range was only linear for concentrations ranging from 0.01 to 100 ng/ml. The linear regression equation was $y=11.836x+136.06$ ($R^2=0.9755$, n=5) and the RSD was 12.5; the LOD derived from this equation was 3.16 ng/ml. From the slopes of the equations, it is evident that the sensitivity was improved by a factor of 115 (from 0.1029 to 11.836 RU) per concentration unit (ng/ml) by the AuNPs enhanced immunosensing. The result is also consistent with other previous studies [11]. Similarly, the linear regression equation for the double enhancement based on gold and

enzyme precipitation was y=43.24x+ 309.24 (R^2=0.9826, n=6). The detectable range of concentration was obtained over 0–100 ng/ml. Moreover, fluctuation of RU values at zero concentration of the analyte was invariable in the enzyme precipitation reaction, leading to a very low RSD being achieved (RSD=0.4). Consequently, the LOD was found to be as low as 0.027 ng/ml of the PSA/ACT complex (or LOD=300 fM). As a result, the SPR sensitivity was improved by a factor of 3.65 in comparison with the AuNPs enhanced assay (from 11.836 to 43.24 RU), or by a factor of 420.2 in comparison with the primary immune response (from 0.1029 to 43.24 RU) per concentration unit (ng/ml).

Several methods have been implemented to achieve better sensitivity of PSA detection based on SPR [4, 7, 13]. However, the detections were limited at nM scale. In this study, a three orders of magnitude enhancement has been achieved for the SPR immunosensing (0.027–56.9 ng/ml). The detection limit of the assay was found to be as low as the femto molar level after the double enhancement. The results show that by functionalizing the AuNPs with HRP, we can exploit both outstanding characteristics of gold colloid and enzyme precipitation sequentially in a so-called double enhancement strategy. Although simple in design and concept, it moreover also provides a fast, highly sensitive, and wide-range analytical method for detecting not only the PSA/ACT complex, but also other analytes at low concentrations.

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