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# Label-Free Immunosensing for α-Fetoprotein in Human Plasma using Surface Plasmon Resonance

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## 1. Introduction

Surface plasmon resonance (SPR)-based sensing has been widely used for studies on biomolecular interactions (1). It is well-known that the principle of the SPR sensing is based on the detection of a small refractive index change on a thin metal film caused by complex formation between biomolecules such as antigen/antibody complexes (2). The SPR-based immunoassay has been examined to expand its application to the clinical diagnosis for the detection of biomarkers in low concentration in human blood such as tumor markers (3) and cardiac failure markers (4). The characteristics of the SPR sensing are a label-free detection without fluorescent dyes and a real-time monitoring. In addition, the SPR-based sensing devices could be miniaturized or portable, and expected to be applied to the point-of-care testing, i.e. bedside determination, due to its simple optical setting. Therefore, it is important to detect biomarkers in low concentration in blood accurately and rapidly by the SPR-based sensing.

In this study, the concentration of α-fetoprotein (AFP) in human plasma was determined by the SPR method, because it was the one of the major tumor markers for hepatocellular tumors. The normal concentration of AFP in plasma is less than 20 ng/mL, but its level markedly increases to about 700 ng/mL during the disease state. The method developed for detection of AFP is expected to be applicable to other tumor markers. Then, we also tried to measure the AFP by using miniaturized SPR imaging apparatus. In the SPR imaging apparatus where CCD camera was used as the detector, multiple biomolecular interactions can be in real time studied on the same array. Therefore, various tumor markers would be measured in parallel.

## 2. Methods

### *2.1. SPR instruments*

A chromium layer 1.0nm in thickness was deposited on the surface of BK7 glass plate, followed by the deposition of a 49 nm thick gold layer. Gold-coated glass plates were immersed in a TEG and HEG-mixed (9/1, by molar ratio) ethanol solution for 24 hr, and then the TEG and HEG-mixed SAM was formed on the surface of the gold-coated glass plates. For the measurement of AFP in human plasma we employed both an in-house designed SPR instrument and SPR imaging instrument with Kretschmann configuration. The SPR instrument comprises a flow cell made of a glass plate with a SAM set on a hemicylindrical prism of the SPR instrument. The beam of a HeNe laser (λ = 632.8 nm) was linearly *p*-polarized and then irradiated through the hemicylindrical prism to the back side of the glass plate. The intensity of the reflectance was measured by a photodiode detector. The SPR imaging apparatus comprises super luminescence diode (SLD) laser beam (λ = 905 nm) and glass plate set on the triangular prism. The intensity of the reflectance from the gold layer on the glass plate was monitored by CCD camera (TERAMECS Co.,LTD.). Sample and buffer solutions (33 mM phosphate buffer, pH6.6) were allowed to pass through the flow cell at 4.0 mL/min, with all measurements performed at 30 $^{o}$C.

### *2.2. SPR-based immunoassay for AFP detection*

The monoclonal antibody was covalently immobilized to the TEG and HEG-mixed SAM surfaces as follows: 0.1 M EDC and 0.05 M NHS in phosphate buffer were flowed to activate the COOH groups for 15 min and then the monoclonal antibody solution (10 μg/mL) was flowed for 25 min. To suppress the nonspecific adsorption, a BSA solution (10 mg/mL) was flowed. Then a solution of AFP or human plasma (obtained from healthy donors) containing AFP (50 to 500 ng/mL) was flowed, followed by the flowing of 0.05% tween 20 in PBS solution and then the secondary antibody (10 μg/mL, polyclonal) was applied sequentially. For the detection of AFP in human plasma, a solution of anti-rabbit IgG antibody (10 μg/mL, polyclonal) was finally flowed for the SPR signal enhancement. The same procedures were performed in the absence of AFP in the buffer or human plasma.

For the SPR imaging apparatus, AFP and anti-AFP antibody were simply mixed for forming AFP/AFP antibody complex and sample and the solutions were allowed to pass through the flow cell on the sensor surface. After AFP (0 - 500 ng/mL) and anti-AFP antibody (10μg/mL) were reacted for 30 min at room temperature, the sample were applied to the sensor surface carrying primary antibody.

## 3. Results and Discussion

Figure 1 showed the SPR profiles of sequential reactions for AFP detection in human plasma containing 500 ng/mL AFP. After immobilization of primary antibody and blocking treatment, plasma containing AFP was perfused. Then, tween 20 in PBS and secondary antibody were sequentially flowed. As shown in Fig. 1(a), the SPR signal was hardly increased during the flow of plasma containing 500 ng/mL AFP to the sensor surface carrying primary antibody. The variation of the SPR signal changes caused by AFP bonding were hided by the large refractive index change due to the presence of plasma component (bulk effect) and non-specific adsorption of plasma proteins on the sensor surface even after washing with a tween 20 solution. When a solution of secondary antibody was applied after washing with the tween 20 solution, an SPR signal shift (155 mDA) was clearly observed as shown in Fig 1(a). Although small amount of serum proteins (110 mDA) was remaining on the surface even after washing with the tween 20 as shown in Fig. 1(a), this did not interfere with the detection of the binding of secondary antibody. The SPR signal shift was further enhanced by applying anti-rabbit IgG antibody (polyclonal) against the secondary antibody as shown in Fig. 1(b), including SPR signal shifts, when 50 ng/mL AFP solution and a solution without AFP were examined. As is known, plasma from healthy persons contains small amount of AFP, < 20 ng/mL. The effect of this small amount of AFP on the SPR signal shift was carefully examined. The SPR method in conjunction with usage of secondary and third antibodies allowed us to detect small amount of AFP in plasma from healthy persons.

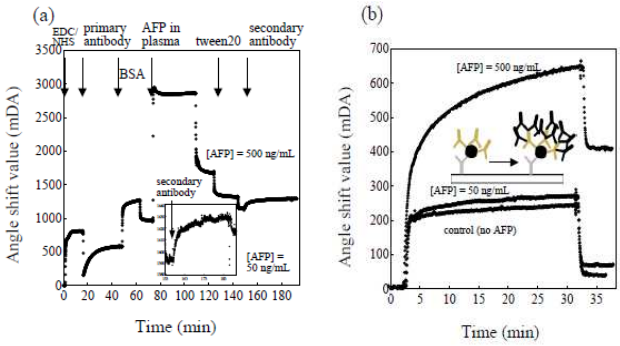


**Fig. 1** SPR-based immunoassay for AFP in human plasma. (a) SPR profiles of sequential reactions for AFP detection in human plasma containing 500 ng/mL AFP. (b) Time course of SPR profiles caused by binding of polyclonal antibody against the secondary antibody ([AFP] = 0, 50 or 500 ng/mL).

Figure 2 showed the image of sensor surface monitored by CCD camera using miniaturized SPR imaging apparatus. When buffer solution was allowed to pass through the flow cell, three lines of gold patterned on the glass plate was darken, indicating the occurring of SPR phenomenon on the gold lines. Figure 3 showed the SPR profiles of detection for the binding of AFP and antibody complex ([AFP] = 0 to 500 ng/mL). Before application into the sensor surface, AFP and secondary antibody were previously reacted at the room temperature for 30min. The reflectance intensity gradually increased with time and reach equilibrium states, indicating that AFP could be detected by the SPR imaging apparatus.

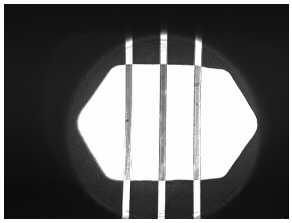

**Fig.2** SPR image of gold patterning (length:16mm, width:200μm) on the glass plate (BK7).

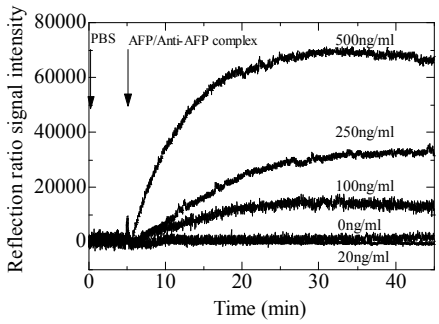


**Fig. 3** SPR plofiles of binding of AFP/antibody complex

## 3. Conclusions

We developed a SPR-based immunoassay sensor to detect AFP in plasma at the nanogram level, which is required by the clinical diagnosis. The results demonstrated that the SPR method is a promising clinical diagnostic tool to determine plasma levels of AFP.


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