

P-11-2

Speedy and Quantitative Analysis of alpha fetoprotein in human plasma with Surface Plasmon Field Enhanced Fluorescence Spectroscopy

Hiromi Takiguchi^{1,2}, Yuji Teramura³ and Hiroo Iwata^{4*}

¹University of Tokyo, Safety and Health Office, School of Engineering, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan

²Advanced Software Technology and Mechatronics Research Institute of Kyoto,
134 Minamimachi Chudoji, Shimogyo, Kyoto 600-8813, Japan

³Kyoto University, Department of Polymer Chemistry, Graduate School of Engineering, Nishikyo, Kyoto 615-8510, Japan

⁴Kyoto University, Institute for Frontier Medical Sciences, 53 Kawara-cho, Shogoin, Sakyo, Kyoto 606-8507, Japan
Phone: +81-75-751-4119 E-mail: iwata@frontier.kyoto-u.ac.jp

1. Introduction

The measurement system composed of Surface Plasmon Resonance (SPR), resonant excitation of the surface plasmon waves at metal surface (typically gold), is sensitive to a small refractive index change caused by complex formation between biomolecules such as antigen-antibody complex. Various systems and apparatuses with SPR are investigated and developed for studies of biomolecule interactions and detection of trace element in human blood by signal amplification [1]. On the other hands, the enhanced optical (electrical) field radiated by coupling of surface plasmon and evanescent was effectively used for excitation fluorophore near the metal surface. This is named surface plasmon field enhanced fluorescence spectroscopy (SPFS, Figure 1(a)), and the sandwich type immunoassay using the secondary antibody conjugated fluorescent probe were measured by SPFS. When incident angle approaches the SPR angle, the reflectance is strongly fallen and the field enhancement factor (FEF) the metal/water interface strongly increases (Figure 1 (b)). Peak of the FEF relative

to incoming light can reach ca. 16 for the system of water/SAM (1 nm)/gold (49 nm)/ S-LAL10 ($n=1.72$) at $\lambda=635$ nm. The SPFS has the character similar to SPR measurement such as real time monitoring of surface event independently and high sensitivity detection by effectively excitation caused by enhanced electrical field. Developing the quantitative method for trace bioactive substance with rapid, simple and high sensitive, are widely employed to home and self-testing.

In this study, we demonstrated the quantitative analysis of alpha-fetoprotein (AFP) in human plasma, which was major as tumor marker for hepatocellular tumors, with SPFS based immunoassay in rapidly and simply with LODs of sub-ng/mL order. Moreover, the time of analysis is necessary to be short (more than 30 minutes of requirement in laboratory test) and as simply as possible. For the achievement of objective, we tried to optimize the condition of SPFS based one-step immunoassay, that were the method to incubate the secondary antibody and sample solution outside the SPR/SPFS apparatus, and quantitative analysis of AFP in human plasma.

2. Method

2.1. SPR/SPFS apparatus

The SPFS with SPR measurement system was based on Kretschmann configuration with simple components [2]. The p-polarized light ($\lambda=635$ nm) was introduced to the triangular prism (S-LAL10, $n=1.72$) and the glass plate with a thin gold film (49 nm) and COOH-terminated SAM on the gold surface was coupled to a triangular prism (S-LAL10) with an index matching fluid ($n=1.72$). For SPR measurement, the intensity of reflected light was monitored by photodiode to set to the appropriate angle in sensor surface modification process. For SPFS measurement, the fluorescent image on the sensor surface was collected by an objective lens, through the interference filter ($\lambda=670$ nm) and acquired by CCD camera with charge multiplier and the image was monitored and the intensity of fluorescent by the laboratory-made intensity scanning software.

2.2 SPFS-based immunoassay for AFP detection

Before the SPFS measurement, the primary antibody (1D5, mouse monoclonal) for AFP was immobilized on the

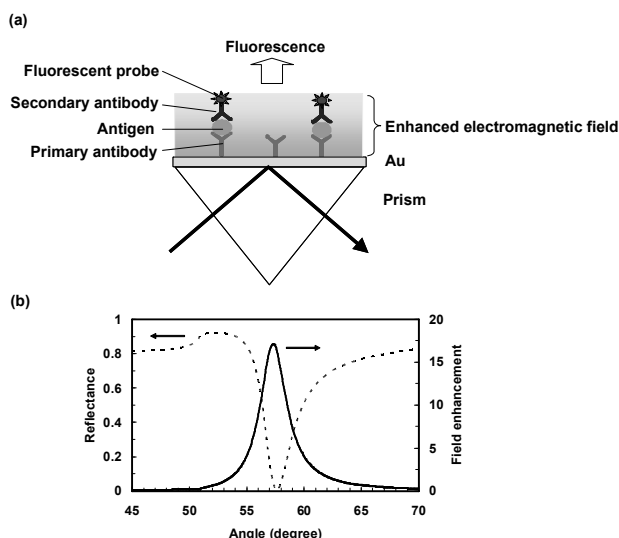


Figure 1. (a) Conceptual drawing of surface plasmon field enhanced fluorescence spectroscopy (SPFS)-based immunoassay. The fluorescent probe conjugated to the secondary antibody is immobilized on the sensor surface as ternary complex and the probe is excited by the enhanced electromagnetic field caused by surface plasmon. (b) The simulation of reflectance and field enhancement on thin gold film in the system of this study.

surface by EDC/NHS coupling method and the blocking treatment by BSA was performed with monitoring SPR signal. In the two-step immunoassay; the sensor surface was exposure AFP standard solution (0-2 ng/mL in PBS) or diluted human plasma, and then the secondary antibody (6D2, mouse monoclonal) conjugated with Alexa Fluor® 647 dye (AF647) solution was flowed in the flow cell. After washing process, the difference intensity between base line signal and the fluorescence intensity was monitored in timing of irradiation. In one-step immunoassay, the sample solution (0-5 ng/mL and diluted human plasma) and secondary antibody conjugated AF 647 was incubated before the SPFS measurement and then the mixture was applied to the sensor surface. The rate of increase of fluorescent intensity (slope of fluorescent change) in the mixture flowing and the fluorescent intensity after washing was monitored. In the all experiments containing surface preparation process were executed at room temperature.

3. Result and Discussion

The conventional sand-witch type (two-step) immunoassay monitored by SPFS method was applied to quantitative analysis of AFP in PBS and human plasma. A typical SPFS sensorgram for two-step assay of AFP (5 ng/mL in PBS) detection was shown in Figure 2. The surface was exposed solutions containing AFP in PBS or 10% human plasma diluted by PBS for 25 min, and then AFP molecule was captured to the immobilized primary antibody. Next, the sensor surface was washed by PBS with 0.05% Tween 20 (5 min) to remove non-specific adsorbed proteins on the sensor surface and the solution of secondary antibody conjugated AF647 was applied for 30 min. The secondary antibody was captured to AFP immobilized on primary antibody and the immune ternary complex was formed. According as forming the complex, the fluorescence signal was gradually increased. Finally, the sensor surface was washed to remove the excess secondary antibody in the flow cell. The fluorescence signal (ΔF) that is the difference between baseline and the fluorescence intensity at 65 min from the sensor surface was monitored. The LOD of two-step immunoassay was 0.07 ng/mL and the quanti-

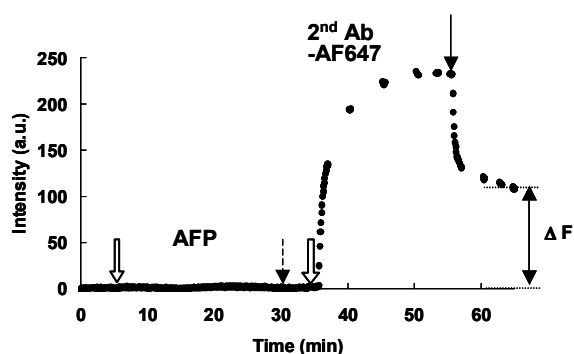


Figure 2. The profile of SPFS-based two-step immunoassay ([AFP]=5 ng/mL in PBS) The difference in intensity from base line is measured. After washing process, the DF is used to determine the concentration of AFP in PBS or human plasma.

tative value as the concentration of AFP in plasma of healthy human was 5.5 ± 0.2 ng/mL determined by standard addition method (5.3 ng/mL determined by ELISA).

To achieve high throughput and short time assay with SPFS measurement, we applied the quantitative analysis of AFP to SPFS-based one-step assay. First, the immune complex of AFP-secondary antibody conjugated AF647 was formed in the sample stock added the antibody before the exposure to the functionalized sensor surface. Second, the complex was exposure to the sensor surface and kinetically adsorbed on the primary antibody. Finally, the sensor surface was washed in order to remove the excess secondary antibody and the ternary immune complex was still retained. The kinetically bonding rate and the fluorescence after washing were monitored to determine the concentration of AFP in PBS or human plasma. We estimated the LODs of the SPFS based one-step immunoassay at 0.2 ng/mL by monitoring of ΔF and 0.3 ng/mL by the slope of fluorescent change, respectively. Applying the quantitative analysis for human plasma with standard adding method, the concentration of AFP in healthy human plasma was 6.0 ± 0.6 ng/mL for the ΔF and 5.2 ± 0.9 ng/mL for the slope, respectively.

4. Conclusion

In this study, we demonstrated the SPFS based immunoassay for quantitative analysis of AFP in human plasma with short time and simple process. The LODs and the ability for quantitation not only satisfied the demand of clinical diagnosis, but the analysis time from the procurement of sample to the end of measurement could be shortened dramatically and the total process in the assay was also available to simplify fundamentally. Moreover, the method can apply to detection of other antigens such as different tumor markers, enzymes or hormones, the performances and advantages of SPFS based immunoassay will supply apparatus for point-of-care-testing with simple and miniaturized composition and easy handling for all users.

Acknowledgements

This study is a part of joint researches, which are focusing on development of basis of technology for establishing COE of nano-medicine, carried out through Kyoto City Collaboration of Regional Entities for Advancing Technology Excellence assigned by Japan Science and Technology Agency (JST).

References

- [1] Y. Teramura, Y. Arima and H. Iwata, *Anal. Biochem.* **357** (2006) 201.
- [2] W. Knoll, H. Park, E-K. Sinner, D. Yao and F. Yu, *Surface Science* **570** (2004) 30