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Effect of dielectric spacer thickness on signal intensity of surface plasmon field-enhanced fluorescence spectroscopy

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ABSTRACT

Surface plasmon field-enhanced fluorescence spectroscopy (SPFS) combines enhanced field platform and fluorescence detection. Its advantages are the strong intensity of the electromagnetic field and the high signal/noise (S/N) ratio due to the localized evanescent field at the water/metal interface. However, the energy transfer from the fluorophore to the metal surface diminishes the fluorescence intensity, and this reduces the sensitivity. In this study, we tested whether polystyrene (PSt) could act as a dielectric layer to suppress the energy transfer from the fluorophore to the metal surface. We hypothesized that this would improve the sensitivity of SPFS-based immunoassays. We used α-fetoprotein (AFP) as a model tumor biomarker in the sandwich-type immunoassay. We determined the relationship between fluorescent signal intensity and PSt layer thickness and compared this to theoretical predictions. We found that the fluorescence signal increased by optimally controlling the thickness of the PSt layer. Our results indicated that the SPFS-based immunoassay is a promising clinical diagnostic tool for quantitatively determining the concentrations of low-level biomarkers in blood samples.

Surface plasmon resonance (SPR)[1] is a sensitive technique that detects changes in the local refractive index near the surface of a thin metal (typically gold) film [1]. It has been used to analyze interactions between biomolecules, like antigen–antibody and ligand–receptor complexes [2]. SPR-based immunoassays provide rapid, label-free detection of various markers [3–8]. In addition, SPR-based sensing devices can be miniaturized and portable; they are expected to be useful in point-of-care testing due to their simple optical requirements. Therefore, SPR-based immunoassays have been investigated for use in clinical diagnoses based on human blood biomarkers, including hormones and tumor-specific molecules [3–8]. We previously described a SPR-based immunoassay for α-fetoprotein (AFP) in human plasma [8]. In that assay, antibody detection could resolve 50 ng/mL AFP. However, plasma from healthy adult donors contains ~3.0 ± 1.9 ng/mL AFP, and an increase in AFP correlated closely with hepatic tumor progression and prognosis [9,10]. Therefore, the clinical diagnosis requires a method that can clearly discriminate between AFP levels on the order of 10 ng/mL. Like AFP, most tumor biomarkers are present in the blood at concentrations that range from nanograms to picograms per milliliter. Thus, the sensitivity of the simple SPR-based immunoassay is insufficient for detecting most tumor biomarkers. Several previous methods have been developed to increase SPR sensitivity, for example, nanobeads that carry a secondary antibody [4,6,7,11]. However, none have provided sensitivity sufficient for clear diagnoses in patients.

Surface plasmon field-enhanced fluorescence spectroscopy (SPFS) combines the enhanced field with fluorescence detection [12]. SPFS offers the advantages of a strong electromagnetic field intensity and the high signal/noise (S/N) ratio due to the localized evanescent field at the metal/water interface. The SPFS-based immunoassay can detect biomarkers at very low levels. In fact, previous studies showed that it could detect tumor biomarkers below the nanogram per milliliter concentration range [13–15]. However, several aspects of this method remain to be optimized to realize the full potential of this highly sensitive diagnostic apparatus. In the current method, the fluorophore transfers energy to the metal surface, which diminishes the fluorescent intensity and results in reduced sensitivity [12,16]. We hypothesized that the sensitivity of the SPFS-based immunoassay could be improved by suppressing this energy transfer.

In the present study, we described a method to suppress the energy transfer from the fluorophore to the metal surface with the use of a polystyrene (PSt) dielectric layer (Scheme 1). We used AFP as a model tumor biomarker in the sandwich-type immunoassay. We determined the dependence of the fluorescent signal intensity on the PSt layer thickness, and compared it to theoretical predictions.

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[1] *Abbreviations used*: AFP, α-fetoprotein; PBS, phosphate-buffered saline; PSt, polystyrene; SPFS, surface plasmon field-enhanced fluorescence spectroscopy; SPR, surface plasmon resonance.

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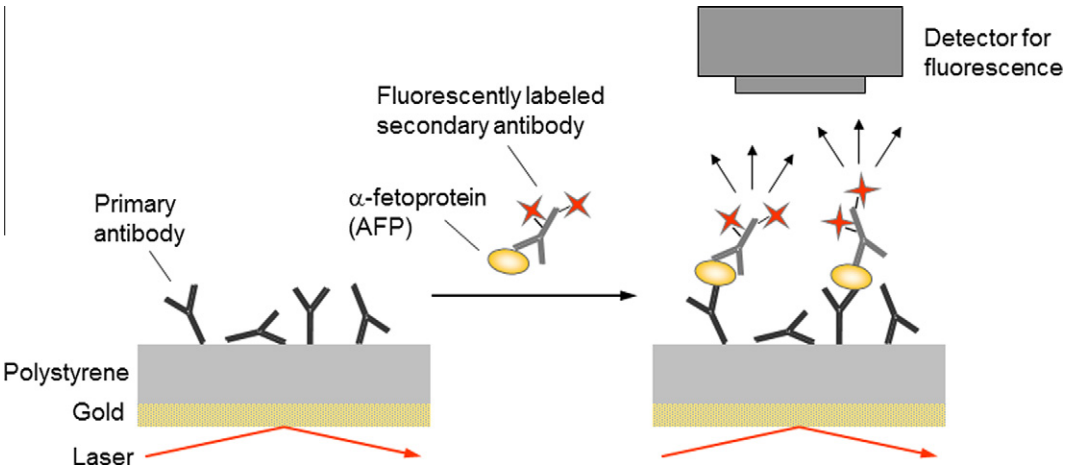

Scheme 1. Schematic illustration of SPFS-based immunoassay for the detection of AFP. (Left) The primary antibody was adsorbed to the sensor surface; (center) the AFP–antibody complex was flowed over the sensor surface; and (right) the primary antibodies captured the AFP complexes and a fluorescence detector quantified the bound signal.

Materials and methods

Reagents

Glass plates (S-LAL10, refractive index: 1.720, 25 × 25 × 1 mm) were purchased from Sigma Koki (Tokyo, Japan). Polystyrene (MW: 125,000–250,000) was purchased from Polysciences (Warrington, PA, USA). Dulbecco's phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Bovine serum albumin, fraction V (BSA), and lysozyme were purchased from Sigma–Aldrich (St. Louis, MO, USA). AFP was purchased from HyTest (Turku, Finland). Mouse monoclonal antibodies against AFP included a primary antibody (clone: 1D5) and a secondary antibody (clone: 6D2), purchased from Mikuri Immunological Laboratories (Osaka, Japan). The secondary antibody and lysozyme were fluorescently labeled with an Alexa647 monoclonal antibody labeling kit and an Alexa647 protein labeling kit, respectively (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Human plasma was drawn from 12 healthy donors and pooled to suppress the variation in donors. Concentration of AFP in the pooled plasma was measured to be 2.4 ng/mL using a chemiluminescent immunoassay (ARCHITECT AFP, Abbott Laboratories. Abbott Park, IL, USA). All subjects enrolled in this research have responded to an Informed Consent, which has been approved and accepted by the ethics review board of Institute for Frontier Medical Sciences, Kyoto University.

Preparation of the sensor chip

S-LAL10 glass plates were cleaned with oxygen plasma treatment in a plasma reactor (PA300AT; O-kuma Engineering, Fukuoka, Japan) under 5 Pa for 1 min. Glass plates were rinsed with deionized water and 2-propanol three times each and dried under a nitrogen gas stream. A thermal evaporation apparatus (V-KS200; Osaka Vacuum, Osaka, Japan) was used to deposit a 1-nm chromium layer, and then a 49-nm-thick gold layer onto the glass plates.

Next, thin PSt layers of various thicknesses were formed on top of the gold-coated glass plates with a spin-coater (1H-DX, Mikasa, Tokyo, Japan). PSt was dissolved in toluene (0.1–1.5%) and then spin-coated onto the plate at 6000 rpm for 60 s. Substrates were stored under vacuum until use.

Characterization of PSt thin films

Static water contact angles on the coated surfaces (PSt film substrates) were determined by the sessile drop method with a contact angle meter (CA-X, Kyowa Interface Science Co. Ltd., Saitama, Japan) at room temperature. A 10-μL water droplet was placed on a substrate, and the contact angle was determined three times. This procedure was repeated at five different places on each surface.

The PSt film thickness was estimated with a home-made surface plasmon resonance apparatus [15]. The S-LAL10 glass plate with the gold and PSt layers was coupled to a triangular prism via immersion oil ($n = 1.720$, Cargille Laboratories, Cedar Grove, NJ, USA). The sample surface was irradiated with a *p*-polarized, He–Ne, laser light ($\lambda = 632.8$ nm) from the air through the prism. The intensity of the reflected light was monitored as a function of the incident angle. The thickness of the PSt layer was determined from the SPR angle shift based on Fresnel's law for a multilayer system of S-LAL10/Cr/Au/PSt/air [17]. The refractive index for PSt was 1.59 [18].

The surface morphology of the PSt thin film was observed with an atomic force microscope (AFM, JSPM-5200; JEOL Ltd., Tokyo, Japan), which was operated with the tapping-mode in air. The AFM images (1 × 1 μm, 512 × 512 pixels) were analyzed with commercial software (WinSPM; JEOL Ltd., Tokyo, Japan) to obtain the arithmetic average roughness (Ra), expressed as:

$$\mathrm{Ra} = \frac{1}{n}\sum_{i=1}^{n}|Z_i - Z_a|, \tag{1}$$

where n is the whole number of pixels, Z_i is the height of the pixel at $n = i$, and Z_a is the average height of the surface.

SPFS apparatus

A SPFS instrument of Kretschmann configuration was fabricated in-house [15], as described by Liebermann and Knoll [12], with minor modifications. Briefly, a glass plate with a thin gold layer was coupled to a triangular prism via immersion oil. A flow cell was prepared on the glass plate by fixing a poly(methylmethacrylate) (PMMA) plate through a spacer made of silicone rubber (thickness: 0.5 mm), and silicone tubes (inner diameter, 0.5 mm;