Evanescent Field in Surface Plasmon Resonance and Surface Plasmon Field-Enhanced Fluorescence Spectroscopies

Sanong Ekgasit,*,¹,‡ Chuchaat Thammacharoen,¹ Fang Yu,¹ and Wolfgang Knoll¹

Sensor Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand, and Max-Planck-Institut für Polymerforschung, Ackermannweg 10, D-55128 Mainz, Germany

The highly sensitive nature of surface plasmon resonance (SPR) spectroscopy and surface plasmon field-enhanced fluorescence spectroscopy (SPFS) are governed by the strong surface plasmon resonance-generated evanescent field at the metal/dielectric interface. The greatest evanescent field amplitude at the interface and the maximum attenuation of the reflectance are observed when a nonabsorbing dielectric is employed. An absorbing dielectric decreases the evanescent field enhancement at the interface. The SPR curve of an absorbing dielectric is characterized by a greater reflectance minimum and a broader curve, as compared to those of the nonabsorbing dielectric with the same refractive index. For a weakly absorbing dielectric, such as nanometer-thick surface-confined fluorophores, the absorption is too small to induce a significant change in the SPR curve. However, the presence of a minute amount of the fluorophore can be detected by the highly sensitive SPFS. The angle with the maximum fluorescence intensity of an SPFS curve is always smaller than the resonance angle of the corresponding SPR curve. This discrepancy is due to the differences of evanescent field distributions and their decay characteristics within the metal film and the dielectric medium. The fluorescence intensity in an SPFS curve can be expressed in terms of the evanescent field amplitude. Excellent correlations between the experimentally measured fluorescence intensities and the evanescent field amplitudes are observed.

Surface plasmon resonance (SPR) spectroscopy has proven to be a powerful affinity biosensor and is becoming an accepted bioanalytical technique for the routine quantification of molecular recognition events, especially those associated with interfaces and thin films.¹² Although prominent applications of the technique are found in life science research, medical diagnostics, nanotechnology, biotechnology, and drug screening, other applications can be found in environmental protection, energy, food safety, and optics. SPR spectroscopy takes advantage of the enhanced evanescent field at the surface of a thin noble metal film for probing thin dielectric film deposited on the metal surface. Near the resonance angle, a strong evanescent field is generated at the metal/dielectric interface by the surface plasmon resonance phenomenon. The exponential decay of the evanescent field makes the SPR signal very sensitive to chemical or physical interactions that can induce thickness and complex refractive index variations near the metal surface. As the refractive index or the thickness of the dielectric film changes, the resonance angle shifts to a new position together with an associated change in the evanescent field amplitude and distribution. The magnitude of the resonance angle shift, the variation of the reflectance at a certain angle of incidence, or both can be correlated to the physicochemical phenomena at the interface. The kinetics of the reactions and binding events as well as properties of the dielectric film can be studied from the observed SPR signals.¹²

In addition to being a rapid and simple sensing technique, the inherent label-free characteristic is the major advantage of SPR spectroscopy. The benefits of this unique feature are widely explored by various studies involve binding events, molecular recognitions, and site-specific interactions at the interfaces;³⁻⁷ however, the intrinsic label-free characteristic imposes limitation on further sensitivity improvement of the technique, especially if the analysis involves small molecules. Small molecules, if binding to surfaces of thin films or to target molecules, induce an insignificant thickness change or refractive index variation, thus inducing an insignificant SPR shift.

To improve the sensitivity and the detection limit of SPR-based bioanalytical techniques, surface plasmon field-enhanced fluores-
cence spectroscopy (SPFS) was developed.3 The technique takes advantage of the strong SPR-generated evanescent field to excite fluorophores near the metal/dielectric interface. The fluorescence emitted by the excited fluorophores can be detected by a CCD camera or by a photomultiplier tube. Since the reflected radiation under conventional SPR setup and the emitted fluorescence under SPFS setup can be detected simultaneously, the optical property, the thickness of the thin dielectric film, or both can be followed via the angular shift of the resonance angle while the physical and chemical changes associated with fluorophores can be monitored via the emitted fluorescence in real time. The combination of the highly sensitive nature of the fluorescence technique with the extremely strong SPR-generated evanescent field makes SPFS exceptionally sensitive to minute changes associated with dielectric films and fluorophores. A detection limit of ~240 pM of human chorionic gonadotropin (hCG) was observed via SPR-based fluoroimmunoassay.4 On the basis of the SPR−SPFS technique, a quantitative and in situ discrimination of single nucleotide mismatches in oligonucleotide double strands was observed.5 A multicomponent macromolecular adsorption onto the surface of functionalized self-assembled monolayers (SAMs) from an aqueous solution was reported.6 Recently, binding events between antibody-bound SAMs and their antigens were studied by the SPR−SPFS technique.7

To effectively utilize the extremely sensitive nature of the SPR−SPFS technique and to design an efficient SPR-based biosensor, the behavior of the evanescent field under various environments must be understood. This paper will show that the strong SPR-generated evanescent field at the metal/dielectric interface governs various unique characteristics of SPR−SPFS spectroscopy. The relationship between the SPFS-emitted fluorescence intensity and the evanescent field amplitude is described. A linear relationship between the absorption of the absorbing dielectric and the evanescent field amplitude is confirmed by the emitted fluorescence.

THEORY

Interaction of Light and Multilayer. A multilayer architecture in an SPR setup (i.e., a thin metal film with multilayer dielectric films) can be represented by a plane-boundary multilayer. The jth layer has a complex dielectric constant, \( \epsilon_j \), and a thickness, \( d_j \); \( j = 1, 2, 3, \ldots, N \). The prism is transparent and has a dielectric constant \( \epsilon_0 \). The semi-ininitely thick dielectric substrate has a complex refractive index, \( \hat{\epsilon}_0 \). The complex dielectric constant of each layer is related to its complex refractive index \( \hat{n}_j \) by \( \hat{\epsilon}_j = \hat{n}_j^2 \) (\( \hat{n}_j = n_j + i \kappa_j \); \( n_j \) is the refractive index, and \( \kappa_j \) is the absorption index). When a parallel-polarized radiation impinges at the prism/multilayer interface with an angle of incidence \( \theta \), the reflectance of the reflected radiation \( R_{ij} \) is given in terms of the complex Fresnel reflection coefficient, \( r_{ij} \), by

\[
R_{ij} = |r_{ij}|^2
\]

where \( r_{ij} = \left( \frac{M_{12} + \epsilon_{20} \epsilon_{10} - (M_{21} + \epsilon_{20} \epsilon_{10}) q_{p}}{(M_{11} + \epsilon_{10} \epsilon_{02} + (M_{21} + \epsilon_{20} \epsilon_{10}) q_{p} + (M_{21} + \epsilon_{20} \epsilon_{10}) q_{p}} \right) \). \( M_{ij} \) is an element of the characteristic matrix \( \mathbf{M} \) of the plane parallel multilayer. The matrix \( \mathbf{M} \) is given in terms of the experimental parameters and characteristics of the multilayer by

\[
\mathbf{M} = \prod_{j=1}^{N} \begin{bmatrix}
\cos(k_{d_j}d_j) & i\sin(k_{d_j}d_j) \\
-iq_j \sin(k_{d_j}d_j) & \cos(k_{d_j}d_j)
\end{bmatrix}
\]

where \( q_j = k_{d_j}/\epsilon_j \) and \( k_{d_j} \) are the z component of the wavevector in the jth layer. \( k_{d_j} \) is given in terms of the x component of the wavevector in the prism \( k_p \) by \( k_{d_j} = \sqrt{\left(2\pi/\lambda\right)^2 \epsilon_j - k_p^2} \) with \( k_p = (2\pi/\lambda) |\epsilon_0 \sin^2 \theta|^{1/2} \).

The time average mean square electromagnetic field at a distance \( z \) from the prism surface within the ith layer is given by the following expressions.8

\[
\langle H_{yz}^2 \rangle = \frac{|U_{iz}|^2}{(2\pi/\lambda)^2} |\mathbf{V}_{iz}|^2, \quad \langle E_{yz}^2 \rangle = \frac{k_p^2}{(2\pi/\lambda)^2} |U_{iz}|^2
\]

\[
\left[ \begin{array}{c}
U_{iz} \\ V_{iz}
\end{array} \right] = \left[ \begin{array}{c}
\cos(k_{d_j}\Delta z) \\ i\frac{\epsilon_j}{k_{d_j}} \sin(k_{d_j}\Delta z)
\end{array} \right] \times \left[ \begin{array}{c}
\cos(k_{d_j}d_j) \\ -i\frac{\epsilon_j}{k_{d_j}} \sin(k_{d_j}d_j)
\end{array} \right] \prod_{j=1}^{N} \left[ \begin{array}{c}
\cos(k_{d_j}d_j) \\ -i\frac{\epsilon_j}{k_{d_j}} \sin(k_{d_j}d_j)
\end{array} \right] \mathbf{H}_i
\]

where \( \Delta z \) is the distance between z and the surface of the \((i-1)\)th layer, \( \langle H_{yz}^2 \rangle \) is the mean square magnetic field in the y direction (yM SM F), \( \langle E_{yz}^2 \rangle \) is the mean square electric field in the x direction (xM SEF), \( \langle E_{yz}^2 \rangle \) is the mean square electric field in the z direction (zM SEF), and \( t_i = 2q_p/[(M_{11} + \epsilon_{10} \epsilon_{02}) q_{p} + (M_{21} + \epsilon_{20} \epsilon_{10}) q_{p}] \) is the complex Fresnel transmission coefficient. \( H_{yz} \) is the magnetic field of the incident radiation, which is given in terms of the corresponding electric field by \( H_{yz} = -e_p^{1/2} E_{yz} \). Note: the plane of incidence is defined by the x–z plane with the x direction as the propagating direction of the radiation (see Figure 1A).

For a simple three-phase system (i.e., prism/metal film/dielectric medium), the electromagnetic field amplitude at the metal/dielectric interface can be given in terms of the complex Fresnel transmission coefficient by the following expressions.

On the metal side:

\[
\langle H_{yz=dx}^2 \rangle = |t_{i0}|^2, \quad \langle E_{yz=dx}^2 \rangle = \frac{e_p^{1/2} k_{20} q_{p}}{2\pi/\lambda |\epsilon_{D}|} t_{i0}^2
\]

\[
\langle E_{yz=dx}^2 \rangle = \frac{k_{sp}}{2\pi/\lambda |\epsilon_{D}|} t_{i0}^2
\]
fluorophore-labeled antibodies. For the SPR drawings of the fluorophore-labeled antibody-bound SAM employed glycol-terminated thiol and (b) biotin-terminated thiol. (C) Schematic Structures of thiols employed for mixed SAM fabrication: (a) ethylene

Absorption is the result of the interaction between the electromagnetic wave and an absorbing medium (i.e., that with a nonzero imaginary part of the dielectric constant). The attenuation of the reflectance is due to absorption by all absorbing media in the system (i.e., the metal film and the absorbing dielectrics). The reflectance can be expressed in terms of the mean square electric field and the dielectric constants of the multilayer by the following expression,

\[ R_p = 1 - A_p = 1 - \left( \frac{2\pi}{\lambda} \sum_{j=1}^{N} \int_{0}^{d_j} \text{Im}[\varepsilon_j](E_{ij}^2) \, dz \right) \]

where \( A_p \) is the absorption in absorbance units and \( (E_{ij}^2) \) is the mean square electric field with parallel polarization (p-M MSEF) at distance \( z \) from the prism surface. The p-M MSEF is the summation of the mean square electric field in the \( x \) direction and that in the \( z \) direction (i.e., \( (E_{ij}^2) = (E_{xj}^2) + (E_{zj}^2) \)).

EXPERIMENTAL SECTION

Instrumental Setup for SPR and SPFS Measurements. A schematic illustration of an SPR—SPFS setup in the Kretschmann—Raether ATR configuration is shown in Figure 1. For an SPR measurement, radiation from a HeNe laser with a wavelength \( \lambda = 632.8 \) nm (Uniphase, Los Angeles) is modulated by a chopper. The plane of polarization and intensity of the modulated radiation are adjusted by two Glan-Thompson polarizers (GT-PI). The sample cell is mounted on a computer-controlled goniometer. A precise angular rotation of the goniometer is controlled via a homemade program. The SPR signal is collected as a function of the angle \( \theta \) defined with respect to the direction normal to the prism/metal interface. For an SPFS measurement, the emitted fluorescence is collected from the backside of the triangular prism. The fluorescence light is focused by a lens (\( f = 50 \) mm, Owis, Germany) and passed through an attenuator and an interference filter (\( \lambda = 670 \) nm, \( \Delta \lambda = 10 \) nm, L0T, 80% transmission) before impinging onto a photomultiplier tube PMT (Hamamatsu, Japan). The photomultiplier tube is connected to a photon counter unit (Agilent, U.S.A.). A neutral filter (i.e., an attenuator) is used to attenuate the fluorescence intensity in order to keep the signals from the photomultiplier tube within its linear range (i.e., <1 x 10⁶ counts/s (cps)). It attenuates the intensity by a factor of 60.88. The factor is obtained from an independent calibration experiment.

The SPFS curve is collected simultaneously with the SPR curve as a function of the measured incidence angle \( \theta_p \). For a triangular prism, the measured incidence angle \( \theta_p \) from the goniometer is different from the actual angle of incidence \( \theta \) at the prism/metal interface due to the refractive index difference between air and the prism, and this difference needs to be accounted for.

relationship between both angles is given by \( \theta_{\text{e}} = \sin^{-1}\left[\left(\frac{n_{\text{Prism}}}{n_{\text{Air}}}\right) \sin(\theta - \varphi)\right] + \varphi \), where \( \varphi \) is the angle at the base of the prism.

Preparation of Substrate and Mixed Self-Assembled Monolayer. LASFN9 slides (Schott Glas, Germany) were cleaned and coated with a \( \sim 50\text{-nm} \) gold film via a commercially available thermal evaporation instrument (Edwards, England). A solution of mixed thiols, ethylene glycol-terminated thiol, and biotin-terminated thiol, with a net thiol concentration of 500 \( \mu\text{M} \), was prepared in absolute ethanol. A mole fraction of the biotin-terminated thiol of 0.04 was employed. The chemical structures of the employed thiols are shown in Figure 1. The self-assembled monolayer (SAM) of mixed thiols was fabricated by immersing the freshly gold-coated LASFN9 substrates in the mixed thiol solution overnight at room temperature. The substrates were rinsed thoroughly with absolute ethanol, blown dry with a stream of nitrogen, and kept under argon environment until being used.

Materials. Sodium dodecyl sulfate (SDS) and phosphate-buffered saline (PBS) tablets were purchased from Sigma-Aldrich. An HBS-EP buffer (degassed 10 mM HEPES-buffered saline, pH 7.4, 150 mM NaCl, 3 mM EDTA with 0.005% (v/v) surfactant P-20; Biacore, Sweden) was employed for the preparation of the protein solutions. The anti-biotin mouse monoclonal antibody 2F5 (isotype IgG1, kappa), the Alexa Fluor 647-labeled rabbit anti-mouse IgG (dye-to-protein ratio of 4.8), and the Alexa Fluor 647 monoclonal antibody labeling kit were purchased from Molecular Probes. Proteins were stocked at micromolar concentration in order to avoid concentration depletion (i.e., absorption of protein to surfaces of the container, tube, or sample cell). The 2F5 antibody was labeled with Alexa Fluor 647 dye by following a standard protocol provided by Molecular Probes. The dye-to-protein ratio was 4.4 as determined by a spectroscopic approach using absorption at \( \lambda = 647 \text{ nm} \) (Alexa Fluor 647) and \( \lambda = 278 \text{ nm} \) (antibody) in PBS buffer. For simplicity, the fluorophore-labeled rabbit anti-mouse IgG is represented by AF-RaM, whereas the fluorophore-labeled anti-biotin mouse monoclonal antibody 2F5 is represented by AF-2F5.

All experiments were performed at room temperature (\( 21 \pm 2 \text{ °C} \)) with HBS-EP as a buffer solution. An aliquot of 1 mL of the sample solution was injected into the flow cell. The sample was left in the flow cell for 15 min to allow a complete binding on the surface of SAM. The cell was then flow-washed and filled with running buffer to avoid bulk solution effects during the SPR–SPFS experiments. The working concentrations of 2F5, AF-2F5, and AF-RaM were 20, 20, and 33 nM, respectively. The antibody-bound SAM could be regenerated by an SDS solution (5 mg/mL in HBS-EP). Once the generation was completed, further binding events could be performed. Schematic illustrations of layer architectures of the antibody-bound SAM are shown in Figure 1.11

RESULTS

Evanescent Field Distribution. A conventional angle-scan SPR curve of a three-phase system calculated by the Fresnel equation is shown in Figure 2. The curve has a critical angle of \( \theta_{\text{c}} = 46.28^\circ \) and a resonance angle of \( \theta_{\text{res}} = 51.39^\circ \). The critical angle, whose value is governed by the refractive indexes of the prism and the dielectric medium, indicates the onset of the attenuated total reflection (ATR) phenomenon. A small reflection loss due to ATR absorption is observed as a baseline of the SPR curve. The resonance angle, on the other hand, indicates the angle at which the wavevector of the incident radiation matches that of the surface plasmon wave and the maximum reflection loss occurs.

At an angle of incidence greater than the critical angle, there are two evanescent fields generated on both sides of metal surfaces: a weak ATR-generated evanescent field at the prism/metal interface and a strong SPR-generated evanescent field at the metal/dielectric interface. Both evanescent fields are strongest at their respective interfaces and decay exponentially as a function of distance from the interfaces. The ATR-generated evanescent field decays as a function of distance from the prism/metal interface into the metal film. The SPR-generated evanescent field, on the other hand, decays as a function of distance from the metal/dielectric interface into both the metal film and the dielectric medium. Near the resonance angle, the evanescent field is strongest at the metal/dielectric interface. At angles far away from the resonance angle, the ATR-generated evanescent field is the only field present.10

The evanescent field consists of the evanescent magnetic field and the evanescent electric field. For the parallel-polarized radiation, the electric field has two components: those in the \( x \) direction and those in the \( z \) direction. Contour plots of the evanescent fields associated with the SPR curve in Figure 2 are shown in Figure 3 (the corresponding 3D-surface plots are available in the Supporting Information). The mean square electric field (\( \mu \text{SMF} \)) and the mean square electric field in the \( x \) direction (\( x\text{-M SEF} \)) are continuous at the metal/dielectric interface. The mean square electric field in the \( z \) direction (\( z\text{-M SEF} \)) is discontinuous at the interface. The \( z\text{-M SEF} \) within the dielectric medium is much greater than that in the metal film due to optical enhancement via the difference of dielectric constants at the interface. A smaller \( |\tilde{E}_0| \) compared to \( |\tilde{E}_| \) makes the \( z\text{-M SEF} \) on the dielectric side greater than that on the metal side (see eqs 5 and 6). Since the p-MSEF is the summation of the \( x\text{-M SEF} \) and the \( z\text{-M SEF} \), it is also discontinuous at the interface. Within the metal film, the \( x\text{-M SEF} \) is greater than the \( z\text{-M SEF} \), whereas the opposite phenomenon is observed within the dielectric medium. The strong SPR-generated evanescent field at the metal/dielectric interface concentrates near the resonance angle. From the contour plots, the existence of the weak ATR-generated evanescent field can be seen near the prism/metal interface (i.e., at a distance close to 0). Due to the difference of the dielectric constants of the metal film and that of the dielectric medium, the evanescent fields in both media decay with different

\[ \theta_{\text{c}} = 46.28^\circ \]

\[ \theta_{\text{res}} = 51.39^\circ \]

\[ |\tilde{E}_0| < |\tilde{E}_| \]

\[ x\text{-M SEF} > z\text{-M SEF} \]

\[ x\text{-M SEF}, z\text{-M SEF} \]
As the distance from the metal/dielectric interface increases, the angle with the maximum evanescent field within the dielectric medium slightly shifts toward a smaller angle. This is due to the angle-dependent nature of the decay constant of the evanescent filed. The evanescent field in the dielectric medium can be expressed in terms of the field amplitude at the metal/dielectric interface and the penetration depth. A greater penetration depth at a smaller angle of incidence makes the evanescent field decays slower. The decay pattern of the evanescent field in the metal film is more complicated due to the absorption of the metal film and the convolution of the ATR-generated and the SPR-generated evanescent fields. Within the metal film, the angles with maximum x-MSEF, on the other hand, shifts toward a greater angle under the same condition (Figure 3B).

The evanescent fields on both sides of the metal/dielectric interface have the same angle with maximum field amplitude, except the p-MSEF. The angles with maximum evanescent fields at the metal/dielectric interface for y-MSMF, x-MSEF, and z-MSEF are 50.91°, 51.14°, and 50.94°, respectively. Due to the difference between the angle with maximum field amplitude of the x-MSEF and z-MSEF, the p-MSEF at the metal/dielectric interface has two maximums. The p-MSEF on the metal side has a maximum at 51.11°, whereas that on the dielectric side (with a greater amplitude) has a maximum at 50.96° (see Figure 4).

**SPR of a Nonabsorbing Dielectric.** A contour plot of the reflectance of a three-phase system (prism P/metal film M/nonabsorbing dielectric D: \( n_p = 1.845, n_M = 0.300 + i3.576, n_D = 45 \text{ nm} \)) is shown in Figure 5A (the corresponding 3D-surface plot is available in the Supporting Information). If the refractive index of the nonabsorbing dielectric \( n_D \) increases, the resonance angle shifts toward a greater angle. Within the range of the refractive index shown in the figure, the resonance angle increases linearly with the refractive index of the dielectric medium. A slight broadening of the SPR curve is observed at high refractive index. A narrow separation between contour lines at the angle smaller than the resonance angle indicates a rapid decrease of the reflectance, whereas a large separation at the angle greater than the resonance angle indicates the opposite phenomenon (see Figure 2). The reflectance minimum is slightly increased as the refractive index increases. The resonance angles and the reflectance minimum at both ends of the refractive index are \( (\theta_{spr} = 51.386°, R_M = 0.030) \) and \( (\theta_{spr} = 59.388°, R_M = 0.035) \) for \( n_D = 1.333 \) and \( n_D = 1.450 \), respectively.

Since the metal film is the only absorbing medium in the system, the attenuation of the reflectance can be assigned to the absorption of the metal film. The absorption is given in terms of the p-MSEF and the imaginary part of the dielectric constant of the metal as \( A = (2\pi/\lambda)^2 |Im(c_x) / k_0| \int_0^{dM} (E_x^2) \, dz \). Near the resonance angle, the weak ATR-generated evanescent field gives only a small contribution to the absorption. The SPR-generated evanescent field, on the other hand, is very strong and contributes almost all of the absorption. Figure 5B shows a contour plot of

**Figure 3.** Contour plots of evanescent fields associated with the SPR curve in Figure 2: (A) y-MSMF, (B) x-MSEF, (C) z-MSEF, and (D) p-MSEF. The solid lines at 45 nm indicate the field amplitude of the incident radiation. The numbers indicate the field amplitudes whose values are normalized by the electric field amplitude of the incident radiation.

**Figure 4.** The evanescent fields at interfaces of the contour plots in Figure 3: (A) y-MSMF, (B) x-MSEF, (C) z-MSEF, and (D) p-MSEF. The dotted lines indicate evanescent fields at the prism/metal interface (on the metal side, \( z = 0^° \)). The solid lines indicate evanescent fields at the metal/dielectric interface (on the metal side, \( z = d_M \)). The broken lines indicate evanescent fields at the dielectric medium (on the dielectric side, \( z = d_D \)). The numbers indicate the angles of evanescent field maximums. Note: The y-MSMFs and the x-MSEFs on both sides of the metal/dielectric interface are the same.
the p-MSEF at the metal/dielectric interface (on the metal side). As the refractive index of the nonabsorbing dielectric increases, the p-MSEF near the resonance angle slightly decreases. Although the field at the interface decreases, the term \( \langle 2 \pi / \lambda \rangle^2 \langle E_z \rangle^2 / k_p \) at the resonance angle is relatively constant due to the angle-dependent wavevector \( k_p \) (Figure 5C). This characteristic makes the reflectance minimum stay relatively unchanged as the refractive index of the bulk dielectric increases. The absorption of the metal film equals 0.970 and 0.965 for \( n_D = 1.333 \) and \( n_D = 1.450 \), respectively.

In most SPR sensor applications in which binding events at the surface of the dielectric film are of interest, the events can be modeled by a four-phase system in which the SPR curve is altered by an increment of the dielectric thickness. Figure 6 shows contour plots of the reflectance and the p-MSEF at the metal/dielectric interface (on the metal side) of a four-phase system (prism P/metal film M/nonabsorbing dielectric film DF/nonabsorbing dielectric substrate D: \( n_P = 1.845, n_M = 0.300 + i3.576, d_M = 45 \text{ nm}, n_{DF} = 1.450, n_D = 1.333 \)). The corresponding 3D-surface plot is available in the Supporting Information. As the thickness of the dielectric film increases, the resonance angle shifts to a greater value while the SPR curve is slightly broadened. Within the observed region, the resonance angle increases linearly with the thickness of the nonabsorbing dielectric film. As the thickness of the dielectric film increases, the reflectance minimum slightly increases. Under the observed region, the resonance angles and the reflectance minimum are \( \theta_{SPR} = 51.386^\circ \) and \( R_0 = 0.930 \) and \( \theta_{SPR} = 56.424^\circ \) and \( R_0 = 0.032 \) for \( d_{DF} = 0 \text{ nm} \) and \( d_{DF} = 50 \text{ nm} \), respectively.

**SPR of an Absorbing Dielectric.** Figure 7 shows SPR curves and contour plots of the reflectance and the p-MSEFs at the metal/dielectric interface of a three-phase system (prism P/metal film M/absorbing dielectric AD: \( n_P = 1.845, n_M = 0.300 + i3.576, d_M = 45 \text{ nm}, n_{AD} = 1.333 + i k_{AD} \)). Although the absorption index of the bulk dielectric is small, its SPR curve is significantly different from that of the nonabsorbing dielectric. As the absorption index of the bulk dielectric increases, the SPR curve becomes broader while the resonance angle shifts slightly toward a greater value and becomes less obvious (Figure 7A). The critical angle is also obscured due to the absorption by the dielectric. The p-MSEFs at the interface (on the metal side and on the dielectric side) decrease significantly as the absorption index of the dielectric...
The measured incidence angle 
° had a resolution of 0.1° near the resonance angle.

a A LASF N9 glass (\( \kappa = 1.778 \)) was employed as a coupling prism, and the HBS-E P buffer (\( \kappa = 1.42 \)) was employed as a semi-infinitely thick nonabsorbing dielectric substrate. b Layer with fluorophore (Alexa Fluor 647) labeling. c The measured incidence angle had a resolution of 0.1° near the resonance angle.

Similar to that of the nonabsorbing dielectric, the influence of the thickness variation of an absorbing dielectric film can be modeled by a simple four-phase system (prism/metal film/absorbing dielectric film/nonabsorbing dielectric substrate). A simulation of the system is available in the Supporting Information. As the thickness of the absorbing dielectric film increases, the resonance angle shifts to a greater value while the reflectance minimum increases. The absorption of the absorbing dielectric decreases the evanescent field enhancement at the metal/dielectric interface; the greater the absorption, the smaller the resonance angle shifts to a greater value while the reflectance minimum increases. Due to a significantly smaller evanescent field enhancement at the metal/dielectric interface, the reflectance minimum of the absorbing dielectric (\( k_{AD} > 0 \)) is greater than that of the nonabsorbing dielectric (\( k_{AD} = 0 \)).

When the absorbing dielectric film is placed at various distances from the metal film, the SPR curve and the p-M SEF are altered according to the separation. This phenomenon can be modeled by a five-phase system (prism/metal film/absorbing spacer/absorbing dielectric film/nonabsorbing dielectric substrate). A simulation of the system is available in the Supporting Information. As the thickness of the nonabsorbing spacer increases, the resonance angle shifts to a greater value while the reflectance minimum decreases gradually. This is due to a greater p-M SEF at the metal/dielectric interface as the absorption of the dielectric decreases via a greater separation of the absorbing dielectric film from the metal film.

**SPR and SPFS of Surface-Confined Fluorophore (a Weakly Absorbing Dielectric).** The measured curves of the antibody-bound SAM and the corresponding SPFS curves are shown in Figure 8. As the thickness of the antibody-bound SAM increases, the resonance angle and the fluorescence angle (i.e., the angle with the maximum fluorescence intensity \( \theta_{\text{fluorescence}} \)) shift to a greater value. However, the fluorescence angle is always smaller than the corresponding resonance angle (see the Supporting Information). To determine the dielectric constant and the thickness of each dielectric layer, the measured SPR curves are fitted by the N-phase Fresnel equation. The best-fitted parameters, the resonance angle, and the fluorescence angle are shown in Table 1. Comparisons between the experimentally measured SPR curves and the calculated SPR curves based on the best-fit parameters are also available in the Supporting Information.

When the fluorophore-labeled AF-2F5 is bound onto the surface of the virgin SAM, the resonance angle shifts to a greater value, the reflectance minimum increases slightly while the SPR curve broadens slightly (i.e., Figure 8A(a) and (b), respectively). The corresponding SPFS curves, Figure 8B(a) and (b), respectively, indicate that the virgin SAM does not emit any fluorescence, whereas the fluorophore-labeled antibody emits a fluorescence intensity of 11 591 cps. When the layer of AF-2F5 is removed, the resonance angle retreats to the same position as that of the virgin SAM. The SPR curve of the regenerated SAM (Figure 8A(c)) cannot be graphically differentiated from that of the virgin SAM.

### Table 1. Fitting Parameters of SPR Curves in Figure 8A

<table>
<thead>
<tr>
<th>figure</th>
<th>SPR and SPFS of Surface-Confined Fluorophore (a Weakly Absorbing Dielectric)</th>
<th>experimental results†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPR and SPFS</td>
<td>architecture of the multilayer(^a)</td>
<td>dielectric medium thickness (nm)</td>
</tr>
<tr>
<td>Figure 8A(a)</td>
<td>Au -12.78 + i1.42 51.00</td>
<td>SAM 2.25 1.40</td>
</tr>
<tr>
<td>Figure 8A(b)</td>
<td>Au -12.78 + i1.42 51.00</td>
<td>SAM AF-2F5 2.25 2.1025 + i0.098</td>
</tr>
<tr>
<td>Figure 8A(c)</td>
<td>Au -12.78 + i1.42 51.00</td>
<td>SAM 2.25 4.05</td>
</tr>
<tr>
<td>Figure 8A(d)</td>
<td>Au -12.78 + i1.42 51.00</td>
<td>SAM 2F5 2.25 2.1025</td>
</tr>
<tr>
<td>Figure 8A(e)</td>
<td>Au -12.78 + i1.42 51.00</td>
<td>SAM 2F5 AF-RaM 2.25 2.1025 + i0.094</td>
</tr>
</tbody>
</table>

\(^a\) A LASF N9 glass (\( \kappa = 3.4026 \)) was employed as a coupling prism, and the HBS-E P buffer (\( \kappa = 1.778 \)) was employed as a semi-infinitely thick nonabsorbing dielectric substrate. 

\(^b\) Layer with fluorophore (Alexa Fluor 647) labeling. 

\(^c\) The measured incidence angle had a resolution of 0.1° near the resonance angle.
(Figure 8A(a)). Although the SPR curve of the regenerated SAM exhibits the same resonance angle and reflectance minimum as those of the virgin SAM, its corresponding SPFS curve shows a fluorescence intensity of 732 cps (Figure 8B(c)). As the nonfluorophore-labeled antibody 2F5 is bound onto the surface of the regenerated SAM, the resonance angle shifts to a greater value (Figure 8A(d)). Although there is an insignificant change of the reflectance minimum, the corresponding SPFS curve (Figure 8B(d)) shows greater fluorescence intensity (1919 cps), as compared to that of the regenerated SAM. The fluorescence angle also shifts to a greater value in the same fashion as the resonance angle due to the increased thickness of the antibody-bound SAM. When the fluorophore-labeled AF-RaM is bound onto the 2F5-bound SAM, a maximum fluorescence intensity of 64 894 cps is observed at the greater fluorescence angle (Figure 8B(e)). The resonance angle of the corresponding SPR curve shifts to a greater value. The reflectance minimum increases slightly while a slight broadening of the SPR curve is observed (Figure 8A(e)).

DISCUSSION

In SPR of nonabsorbing dielectrics, the resonance angle is governed by the layer architecture of the system. Any variations in the layer architecture alter the p-MSEF amplitude and distribution and, thus, change the resonance angle. As the refractive index and the thickness of the nonabsorbing dielectric film increase, the maximum p-MSEF decreases slightly while the angle with field maximum shifts to a greater value; however, the integration of the p-MSEF within the metal layer is relatively unchanged due to the angle-dependent nature of the wavevector, k_x (see Figure 5C). As a result, the reflectance minimum is insignificantly changed as the resonance angle shifts to a greater value. The attenuation of the reflectance is governed largely by the thickness of the metal film. At the optimal thickness of the metal film, the reflectance minimum is observed. If the thickness of the metal film exceeds the optimal thickness, the reflectance minimum is increasing while the resonance angle is relatively unchanged. A 3D-surface plot and its corresponding contour plot of reflectance at various thickness of the metal film are available in the Supporting Information.

Near the resonance angle, the SPR-generated evanescent field is much stronger than the ATR-generated evanescent field. Thus, a much larger reflection loss is observed near the resonance angle. According to Figures 2 and 4D, the resonance angle is slightly greater than the angle with maximum p-MSEF at the metal/dielectric interface (on the metal side). The discrepancy is due to the decay characteristics of the p-MSEF within the metal film. The angle with the maximum p-MSEF shifts toward a greater value as the distance from the metal/dielectric interface increases. As a result, the integration of the p-MSEF over the metal film (1/k_x) \int_0^{d_M} (E_x^2) dz has the maximum at a slightly greater angle (i.e., the resonance angle), as compared to that of the p-MSEF at the metal/dielectric interface (on the metal side).

Although the x-MSEF and the z-MSEF are directly derived from the y-MSMF, the angle-dependent nature of the wavevectors k_y and k_x makes the angles with field maximum at the interface of the x-MSEF and the z-MSEF different from that of the y-MSMF (see eqs 5 and 6 and Figure 4). The angles with a field maximum at the metal/dielectric interface are as follows: \theta_{max}(x-MSEF) > \theta_{max}(z-MSEF) > \theta_{max}(y-MSMF). The x-MSEF has equal amplitude on both sides of the metal/dielectric interface (see Figures 3 and 4). The optical enhancement due to the difference of dielectric constants at the metal/dielectric interface makes the z-MSEF in the dielectric medium much greater than that in the metal film. Since the p-MSEF is the summation of the x-MSEF and z-MSEF, the x-MSEF in the metal film is greater than the z-MSEF, whereas the z-MSEF in the dielectric medium is greater than the x-MSEF; the p-MSEF in the dielectric medium is similar to the z-MSEF, whereas that in the metal film is similar to the x-MSEF (see Figure 3). Due to this discrepancy, the angle with maximum p-MSEF on the dielectric side is slightly smaller than that on the metal side (see Figure 4D). The evidence of the difference between the p-MSEF in the metal film and the p-MSEF in the dielectric can be observed in SPR–SPFS experiments.\(^3\) According to eq 7, where absorption is proportional to the integration of the p-MSEF within the absorbing layer, the reflection loss in the SPR curve of a weakly absorbing dielectric is dominated by the absorption of the metal film, whereas the fluorescence intensity of the corresponding SPFS curve is proportional to the absorption of the absorbing dielectric. Due to the smaller angle with maximum p-MSEF at the metal/dielectric interface on the dielectric side, as compared to that on the metal side, and the shift toward a greater angle of the decay profile of the p-MSEF within the metal film, the fluorescence angle is always smaller than the corresponding resonance angle.

An insignificant difference between the SPR curve of the virgin SAM (Figures 8A(a)) and that of the regenerated SAM (Figures 8B(a)) indicates the same p-MSEF distributions within the gold film. This implies that on the basis of SPR observation, both films are the same; however, the corresponding SPFS curve of the regenerated SAM in Figure 8B(c) suggests the presence of residual fluorophore-labeled AF-2F5. The SPR curves suggest that the p-MSEF distribution within the gold film was not disturbed by the very weak absorption of the residual fluorophore-labeled AF-2F5. This evidence also emphasizes the extremely sensitive nature of the SPFS; SPR fails in the determination of minute amounts of absorbed molecules but the corresponding SPFS curve shows strong signals. The fluorescence intensity of the current experimental setup can be improved by simply optimizing the experimental parameters (i.e., by changing or removing the attenuator). According to the employed attenuator, the detected fluorescence intensity can be increased by a factor up to 60. Additional improvement can be achieved by optimizing the layer architecture of the multilayer (i.e., by increasing the thickness of the SAM in order to minimize fluorescence quenching due to the resonance energy transfer phenomenon).\(^7,12\)

For SPR of a nonabsorbing dielectric film, the only source for the attenuation of the reflectance is the absorption of the metal film. Although the thickness of the dielectric film is increasing, the reflectance minimum stays the same while the resonance angle shifts to a greater value (i.e., Figure 8A(c) and (d)). This is due to the relatively unchanged (2\pi/varlambda)^2 (E_x^2)/k_x profile as the thickness of the nonabsorbing dielectric film increases. The decay profiles of the p-MSEF at the resonance angles of SPR curves in Figure 8 are available in the Supporting Information. Since the

p-M SEF integration within the gold film is not significantly altered by the increased thickness of the nonabsorbing dielectric film, the absorption of the gold film at the resonance angle and the reflectance minimum stay unchanged. The absorptions of the gold film and dielectric films calculated from the fitting parameters of SPR curves are available in the Supporting Information.

A greater reflectance minimum in Figure 8A(b) and (e) indicates a smaller absorption of the system when additional absorbing dielectric is present. Due to a slight decreasing of the p-M SEF by the absorbing dielectric, the absorption of the metal film becomes smaller while the total absorption (i.e., absorption of metal film and that of the absorbing dielectric) is smaller than that of the same metal film with a nonabsorbing dielectric. The greater the absorption of the dielectric film, the smaller the attenuation of the reflectance. The p-M SEF with a weakly absorbing dielectric is slightly smaller than that with a nonabsorbing dielectric. This phenomenon implies that the surface plasmon-generated evanescent field is maximized if a nonabsorbing dielectric is employed. Although the fluorophore-labeled dielectric films are very thin, their absorptions are greatly enhanced by the SPR-generated evanescent field and the optical enhancement at the metal/dielectric interface. If the weakly absorbing dielectrics are fluorophores or fluorophore-labeled molecules, the fluorescence signal is an alternative means for measuring the absorption of the dielectric film. SPRS employs the strong evanescent field to excite the fluorophores near the metal/dielectric interface. The SPR-generated evanescent field enhances the absorption (and hence, the emission) of the fluorophore; however, if the fluorophore is placed too close to the metal film, the resonance energy transfer to the metal film quenches the fluorescence. Due to the distance-dependent nature of the resonance energy transfer phenomenon, the fluorescence intensity exhibits strong distance-dependent behavior. The fluorescence intensity decreases substantially as the fluorophores are confined close to the metal surface. A decrease in the fluorescence intensity was also observed when the fluorophore concentration (i.e., dye-to-protein ratio) increases, whereas a red shift of the resonance maximums is observed for some red-absorbing fluorescent dyes when the refractive index of the dye solution increases. However, due to a linear relationship between absorption and emission, the fluorescence intensity of an SPFS curve can be expressed in terms of the p-M SEF by

\[
I_{\text{fluorescence}} = K_{\text{optics}} \left( \frac{2\pi}{\lambda} \right) K_{\text{p}} \int_0^z K_{\text{ET}}(z) \text{Im} \left[ \tilde{\epsilon}_{\text{FID}}(E, \Lambda) \right] dz
\]  

where \( \epsilon_{\text{FID}} \) is the dielectric constant of the dielectric film with fluorophores, \( K_{\text{ET}}(z) \) is the fluorescence quenching factor due to resonance energy transfer and self-quenching by neighboring dye molecules, and \( K_{\text{p}} \) indicates an integration over the thickness of the layer with fluorophores. \( K_{\text{optics}} \) is a constant whose value depends on experimental parameters (i.e., attenuation factor, filter, focusing lens, and the optical windows).

Figure 9 shows comparisons between the measured fluorescence intensities (i.e., SPFS curves in Figures 8B) and the integration of the p-M SEF over the thickness of the layer with fluorophores. The p-M SEF is calculated with the layer architecture obtained from the best-fitted parameters of the corresponding measured SPR curves. In terms of peak shape and the fluorescence angle, excellent agreements between the experimentally measured SPFS curves and the corresponding evanescent field integrations are observed.

Although the amplitude of the p-M SEF near the metal surface is higher than that at a greater distance, a more efficient fluorescence quenching is expected. The fluorophores close to the metal surface emit fluorescence of a much smaller intensity than that at a greater distance, although their absorptions are approximately the same. The same magnitude of reflectance minimums of SPR curves in Figure 8A(b) and (e) implies that the absorptions of fluorophores are approximately the same (an accurate absorbance from the fitting parameters can be found in the Supporting Information). However, the corresponding fluorescence intensity of the surface-confined AF-RaM (Figure 8B(e)) is 5.4 times stronger than that of the surface-confined AF-2F5 (Figure 8B(b)). This is due to a smaller quenching efficiency via a larger separation from the metal surface.

The increase in the fluorescence intensity after a nonfluorophore-labeled 2F5 layer is bound onto the surface of the regenerated SAM is also due to the distance-dependent fluorescence quenching. Because of a small number of the residual AF-2F5

and a longer chain length of the biotin-terminated thiol, as compared to that of the ethylene glycol-terminated thiol, the residual AF-2F5 molecules on the surface of regenerated SAM can fall back and lie on top of the SAM surface due to the lack of a constraint by neighboring molecules and the flexibility of the long-chain biotin-terminated thiol. This phenomenon brings the fluorophore-labeled AF-2F5 molecules closer to the gold surface where the quenching efficiency increases substantially (Figure 8B(c)). When additional nonfluorophore-labeled 2F5 molecules are bound onto the abundantly available biotin-terminated thiol, the residual fluorophore-labeled AF-2F5 molecules are forced to displace at the SAM surface. As the distance of the excited fluorophore from the metal surface increases, the quenching efficiency decreases, and the stronger fluorescence intensity is observed (Figure 8B(d)).

The SPR curve of an absorbing dielectric is more complicated than that of the nonabsorbing dielectric. The curve becomes broader while the reflectance minimum increases as the absorption of the absorbing dielectric increases. The attenuation of the reflectance, which indicates the absorption of the metal film and that of the absorbing dielectric, decreases significantly as the absorption of the dielectric increases. The resonance angle is governed by the refractive index and thickness of the dielectric film, whereas the attenuation of the reflectance is governed by the absorption index of the dielectric film, complex refractive index, and thickness of the metal film. The magnitude of the evanescent field enhancement at the metal/dielectric interface decreases significantly as the absorption of the absorbing dielectric increases. As a result, the total absorption of the system decreases, although the absorption index, the thickness of the absorbing dielectric, or both increase. The difference between the p-M SEFs at the metal/dielectric interface (on the metal side and on the dielectric side) and their decay characteristic also contributes to the broad feature of the SPR curve of the absorbing dielectric.

CONCLUSIONS

The unique characteristics of the SPR and SPFS curves are governed by the SPR-generated evanescent field. The highly sensitive nature of the SPR and SPFS techniques is due to the strong evanescent field enhancement at the metal/dielectric interface. The greatest evanescent field amplitude at the interface is observed when a nonabsorbing dielectric is employed. With an absorbing dielectric, the evanescent field enhancement is significantly decreased (as compared to that of nonabsorbing dielectric). The SPR curve of an absorbing medium is characterized by a broader curve with a high reflectance minimum. For a weakly absorbing dielectric, such as nanometer-thin surface-confined fluorophores, the absorption is too small to induce a significant change in the SPR curve. The only evidence of the absorption is the fluorescence signal from the excited fluorophores. The fluorescence angle in an SPFS curve is always smaller than the resonance angle in the corresponding SPR curve due to the smaller angle with maximum p-M SEF at the metal/dielectric interface on the dielectric side, as compared to that on the metal side, and the shift toward a greater angle of the decay profile of the p-M SEF within the metal film. Although the absorption of the fluorophores near the metal film is greatly enhanced by the strong SPR-generated evanescent field, their fluorescence intensity is substantially attenuated by the quenching phenomenon. Excellent agreement between the p-M SEF integrations over the fluorophore-labeled dielectric layers and the experimentally measured fluorescence intensities from SPFS measurements is observed.

ACKNOWLEDGMENT

S. E. gratefully acknowledges supports from the Thailand Research Fund (TRF Contract No. RSA/07/2545) and the Alexander von Humboldt (AvH) Foundation.

SUPPORTING INFORMATION AVAILABLE

Additional theoretical simulations and Supporting Information for the evaluation of the experimental data. These materials are available free of charge via the Internet at http://pubs.acs.org.

NOTE ADDED AFTER ASAP POSTING

This paper was inadvertently posted before all corrections had been made. Part B of Figure 1 and the text equation at the top of the fourth page were corrected, and the paper was reposted on March 22, 2004.

Received for review November 10, 2003. Accepted February 4, 2004.

AC035326F