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Immunoassays based on directional surface plasmon-coupled emission

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Abstract

We described a new approach to immunoassays using surface plasmon-coupled emission (SPCE). Fluorescence is visually isotropic in space, so that the sensitivity is limited in part by the light collection efficiency. By the use of SPCE, we can efficiently collect the emission and convert it to a cone-like directional beam in a glass substrate. SPCE is the coupling of excited fluorophores with a thin metal film, resulting in radiation of surface plasmons into the higher refractive index media. We used SPCE to develop a model affinity assay using labeled goat anti-rabbit immunoglobulin G (IgG) antibodies against rabbit IgG bound to a 50-nm-thick silver film. Binding of labeled IgG to the surface resulted in increased intensity observed at an angle of 75° from the normal in the glass substrate. The SPCE intensity depends on proximity of the fluorophore to the silver film and does not require a change in quantum yield upon binding. The use of SPCE is shown to provide background suppression because excited fluorophores distant from the silver film do not result in SPCE. Sensitivity and selectivity can be further increased by excitation under conditions of surface plasmon resonance (SPR) because the evanescent field is enhanced by the resonance interaction and excitation is limited to the region near the metal. We believe SPCE will provide a new technology for high sensitivity and selectivity in surface-bound assays and microfluidic systems.

Keywords

Fluorescence immunoassay; Surface plasmon-coupled emission; Silver film; Background suppression

1. Introduction

Fluorescence immunoassays are extensively used in medical diagnostics (Gosling, 1980; Vo-Dinh et al., 1993; Hemmila, 1992; Van Dyke and Van Dyke, 1990). A wide variety of approaches have been used including polarization (Klein et al., 1993; Fiore et al., 1988; Dandliker and de Saussure, 1970), resonance energy transfer (RET) (Morrison, 1988; Ullman et al., 1976) and gated “time-resolved” assays based on long lived lanthanide emission (Diamandis, 1988; Lövgren and Pettersson, 1990; Soini, 1984). There is continued development of new approaches to fluorescence immunoassays, including time-resolved assays (Ozinskas et al., 1993; Nithipatikom and McGown, 1987), RET assays for larger molecular weight species (Bruno et al., 2001) and multi-photon excitation (Baker et al., 2000). In recent years, there has been an emphasis on high-throughput immunoassays using multi-well plates (Schobel et al., 2001) and microparticles (Yang et al., 2001).

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The usefulness of immunoassays depends on their sensitivity and specificity. Sensitivity is typically limited by the background autofluorescence, which is present in all biological samples. Autofluorescence is also found in the optical elements of the instrumentation. In the present report, we describe a new format for immunoassays, which provides increased sensitivity and background rejection by efficient light collection of emission occurring near the bioaffinity surface. The contribution of optical components to the background will also be decreased due to an amplified excitation field, allowing the use of lower incident light intensities.

Our approach is based on the resonance coupling of excited fluorophores with electron oscillations in a thin silver or gold film. These oscillations are called surface plasmons. Excited fluorophores within about 200 nm of a thin film excite the plasmons, which in turn radiate in the glass substrate under the film (Lakowicz, in press; Gryczynski et al., in press). The radiative light appears at a sharply defined angle and displays almost complete p-polarizations. We used this phenomenon in a model immunoassay against rabbit immunoglobulin G (IgG), using fluorescently labeled anti-rabbit IgG (Scheme 1). We showed the surface-bound rhodamine IgG resulted in directional emission into the glass under the metal film and liquid sample. Emission from labeled unbound antibody was suppressed over 100-fold, depending on the optical configuration.

2. Theory

The phenomenon of surface plasmon-coupled emission (SPCE) is relatively unknown, so it is informative to briefly review the principles and the theory. SPCE is closely related to surface plasmon resonance (SPR). The optical properties of surface plasmons have been described in detail (Raether, 1977; Raether, 1988), as have the applications of SPR to measurement of bioaffinity reactions (Salamon et al., 1997; Melendez et al., 1996; Malmqvist, 1999). Surface plasmons cannot be excited from air by incident light, SPR occurs when light is incident on a metal through a higher refractive index medium such as glass. The surface plasmons are only excited at a specific angle of incident (θ_{sp}) where the reflectivity decreases at other angles of incident (θ_i) the reflectivity of the metal is high.

SPR occurs when the wave vector of the incident light, in the metal-glass plane, equals the wave vector of the surface plasmon (k_{sp}). The wave vector of the incident light in the prism is given by $k_p = 2\pi/\lambda = n_p k_0$, where λ is the wavelength in the prism, n_p is the refractive index of the prism and k_0 is the wave vector in a vacuum or air. The in-plane x -component of the wave vector is given by

$$k_{sp} = k_x = k_0 n_p \sin \theta_{sp} \quad (1)$$

where θ_i is measured from the normal axis (Scheme 2), SPR occurs when

$$k_{sp} = k_x = k_0 n_p \sin \theta_{sp} \quad (2)$$

Calculation of the surface plasmon wave vector is more complex. For a metal, the dielectric constant is usually an imaginary number

$$\epsilon_m = \epsilon_r + i \epsilon_{im} \quad (3)$$

where $i = \sqrt{-1}$ and the subscripts indicate the real (r) and imaginary (im) components. These constants are wavelength (frequency)-dependent. Because the real part of ϵ_m is larger than the imaginary part, the wave vector of a metal can usually be approximated by

$$k_{sp} = k_0 \left(\frac{\epsilon_r \epsilon_p}{\epsilon_r + \epsilon_p} \right)^{1/2} \quad (4)$$

While not explicit in Eqs. (1)–(4), SPR only occurs for p-polarized incident light.

SPCE is similar to SPR in reverse. Instead of illumination through a prism, the metal feels near-field interactions with an excited fluorophore, resulting in creation of surface plasmons. These plasmons then radiate into the glass substrate at the surface plasmons angle for the emission wavelength (θ_F). The plasmons radiate at the plasmon angle because this is needed to match the wave vectors. The plasmons cannot radiate into the sample because the wave vectors cannot be matched.

Examination of Scheme 1 reveals that the SPCE device can be illuminated in two ways. The metal can be illuminated from the water side, the reverse Kretschmann (RK), which cannot create surface plasmons. The excited fluorophores near the metal can couple and create SPCE. Since the incident field does not undergo a resonance interaction with the metal the fluorophores are excited nearly equally across the sample. The device can also be illuminated through the prism, called the Kretschmann configuration (KR). When $\theta_i = \theta_{sp}$, there exists an evanescent field above the gold in the sample out to about 200 nm. This evanescent field is enhanced about 40-fold by the resonance interaction (Liebermann and Knoll, 2000; Neumann et al., 2002). Hence KR illumination results in selective excitation near the metal surface. The enhanced field can allow the illumination intensity to be decreased, further reducing the background.

3. Materials and methods

3.1. Reagents

Glass microscope slides (Corning) were vapor deposited with a continuous 50-nm-thick silver layer by EFM (Ithaca, NY).

Rabbit IgG (anti-mouse IgG produced in rabbit, total protein concentration 10 mg/ml, active antibody concentration 2.3 mg/ml) was from Sigma. Rhodamine Red-X-anti-rabbit IgG (produced in goat) conjugate and AlexaFluor647-anti-mouse IgG (produced in rabbit) conjugate (as stock solutions) were from Molecular Probes. Buffer components and salts (such as bovine serum albumin, glucose, sucrose and AgNO_3) were from Sigma-Aldrich.

HPLC purified and concentrated bovine hemoglobin (HbBv) solution (~17%) was kindly donated by Dr. E. Bucci. Absorbance spectra taken at different dilutions of this HbBv solution showed that 1 mm thick layer of non-diluted HbBv solution has optical density (OD) of 5 at 514 nm, the excitation wavelength and OD of 3 at 590 nm the emission maximum of the bound labeled antibody).

3.2. Coating slides with IgG

Slides were non-covalently coated with rabbit IgG: 2 ml coating solution of IgG (30–50 μl of stock solution dissolved in 4 ml Na-phosphate buffer, 50 mM, pH 7.4) was added to the slide, and slide was incubated overnight at room temperature in a humid chamber. Slides were then rinsed with water, washing solution (0.05% Tween-20 in water) and water. Blocking was

performed by adding 2.5 ml of blocking solution (1% bovine serum albumin, 1% sucrose, 0.05% NaN_3 , 0.05% Tween-20 in 50 mM Tris-HCl buffer, pH 7.4) and incubation at 37 °C for 1 h in humid chamber. The slides were rinsed with water, washing solution (0.05% Tween-20 in water) and water, covered with Na-phosphate buffer (50 mM, pH 7.4) and stored at + 4 °C until use.

3.3. End-point binding experiment

Dye-labeled conjugate Rhodamine Red-X-anti-rabbit IgG (stock solution diluted 200 times with Na-phosphate buffer, 50 mM, pH 7.4) was added to the slide (coated with rabbit IgG as described above) and incubated at 37 °C in a humid chamber for 1.5 h. Slide then was rinsed with water, washing solution (0.05% Tween-20 in water) and water. Then, a rubber ring (7 mm diameter and 9 mm height) was placed on the metallic side of the slide and covered with a second glass slide. About 1.5 ml of the Na-phosphate buffer, 50 mM, pH 7.4, was added inside the rubber ring chamber using needle, and fluorescence measurements were performed at two different optical configurations (Kretschmann and reverse Kretschmann).

To test the effect of a background we used two different solutions added inside the rubber ring cell: (1) a highly absorbing HbBv solution as non-fluorescent background or (2) a highly fluorescent solution of non-binding AlexaFluor647-anti-mouse IgG conjugate. The HbBv solution was used undiluted and Alexa-Fluor647-anti-mouse IgG conjugate was used at 500-fold dilution.

3.4. Kinetic binding experiment

A rubber ring (7 mm diameter and 9 mm height) was placed on the surface of the metal mirror slide (coated with Rabbit IgG as described above) and covered with a second glass slide. About 1.5 ml of the dye-labeled conjugate Rhodamine Red-X-anti-rabbit IgG (stock solution diluted 200 times with Na-phosphate buffer, 50 mM, pH 7.4) was added inside the rubber ring chamber using needle. Kinetics was immediately monitored at room temperature.

3.5. Spectroscopic measurements

Absorption spectra were measured on Hewlett Packard model 8543 spectrophotometer using 1 cm cuvettes. Emission measurements in cuvettes were performed using a Varian Eclipse spectrofluorometer. For fluorescence measurements with microscope slides used index-matching fluid to a hemicylindrical prism made of BK7 glass and positioned on a precise rotatory stage equipped with the fiber optics mount on a 15 cm long arm (Gryczynski et al., in press). This configuration allowed fluorescence observation at any angle relative to the incident angle. The outlet of the fiber was connected to an Ocean Optics SD2000 spectrofluorometer for emission spectra and intensity measurements. The excitation was from a pulsed mode-locked argon ion laser (Coherent). Scattered and reflected incident light (514 nm) was suppressed on observation by using a holographic supernotch-plus filter (Kaiser Optical System, Ann Arbor, MI).

4. Results

Our device for the SPCE immunoassay is shown in Scheme 2. The sample was held in a cylindrical volume by an o-ring between two slides, the upper slide being coated with a 50-nm silver film. Rhodamine-labeled IgG was bound near the silver surface by its binding to the surface-bound antigen. Initially, the sample was illuminated in the RK configuration, which does not create surface plasmons in response to the incident light. We measured the emission intensity for all accessible angles from the normal axis (Fig. 1). The intensity observed through the prism was sharply directed near $\pm 75^\circ$. This value is in good agreement with that calculated from minimum reflectance for p-polarized plasmon mode (Gryczynski et al., in press).

The emission spectrum of the SPCE was characteristic of the rhodamine probe (Fig. 2) and spectrum was not corrupted by scattered light at the excitation wavelength. An unusual characteristic of SPCE is the near complete polarization in the p-direction, meaning the electric vector is oriented parallel to the plane of incidence. Fig. 2 shows the emission spectra collected through an emission polarizer oriented p or s. The orientation of the excitation polarizer did not affect these intensities. The p-polarized intensity is 20-fold more intense, resulting in a polarization of $p \approx 0.9$. This large value cannot be the result of photo selection in an isotropic media. Also, this value is independent of the orientation of the excitation polarization. This p-polarization proves that the emission is due to surface plasmons, which under these conditions cannot emit s-polarized light.

We tested the use of SPCE to measure the binding kinetics of the rhodamine-labeled antibodies to the surface bound antigen. Fig. 3 shows the emission intensities after adding labeled antibody. The emission climbs rapidly and reaches a limiting value. It is important to recognize that this 10-fold change in intensity is not the result of a change in the rhodamine quantum yield upon binding. We measured the effect of binding the rhodamine-labeled goat antibody to the antigen while both were free in solution, and found the intensity decrease due to binding was about 25%. This indicates the intensity change is due to localization of the probe in the evanescent field near the silver. Thus the use of SPCE is a generic method to detection of surface localization by a change in intensity, but does not require a change in the fluorophore quantum yield upon binding.

We tested several optical configurations to determine the relative intensities and extent of background rejection possible using SPCE. These three configurations are shown in the right-hand panels in Fig. 4. This sample consisted of the surface saturated with rhodamine-labeled antibody. We then added Alexa 647-labeled antibody (not binding to the surface) to mimic autofluorescence from the sample. The 0.03 μM concentration of this antibody (0.13 μM of Alexa dye) resulted in dominant free-space fluorescence signal from the sample. First the sample was excited using the RK configuration, and the free space emission observed from the same water side of the sample. Compared to subsequent measurements the intensity of the desired rhodamine antibody (below) the signals were weak. The free-space emission was dominated by the emission from Alexa at 670 nm, with only weak rhodamine emission at 595 nm. We then measured the emission spectrum of the SPCE signal (middle panel), while still using RK illumination. The emission spectrum was dramatically changed from a 10-to-1 excess of the unwanted background to a 5-to-1-excess of the desired signal. Hence, the use of SPCE resulted in selective detection of the rhodamine-labeled antibody near the silver film.

We then changed the mode of excitation to the KR configuration (Fig. 4, bottom panel). In this case, the sample was illuminated at Θ_{sp} creating an evanescent field in the sample. The overall intensity was increased 10-fold while further suppressing the unwanted emission from Alexa 647. The increased intensity and decreased background is the result of localized excitation by the resonance-enhanced field near the metal. In this case, the emission was due almost entirely to the rhodamine, with just a minor contribution from the Alexa-labeled protein.

In medical testing, it is often desirable to perform homogenous assays without separation steps, sometimes in whole blood. We reasoned that SPCE should be detectable in optically dense media because this signal arises from the sample within 200 nm of the surface. To mimic whole blood, we added 17% bovine hemoglobin solution (HbBv), which had optical densities of 5 and 3 at 514 and 590 nm, respectively. In a 1.0-mm-thick sample, these optical densities would attenuate the signal about a million-fold. Using SPCE, the signal was attenuated less than three-fold (Fig. 5). These results show the potential of using SPCE in optically dense samples.

5. Discussion

Our results show that SPCE is generally useful for measurement of bioaffinity reactions. The signal is due to fluorophores located close to the bioaffinity surface and binding can be detected without separation steps. Importantly, binding can be detected without any change in quantum yield of the fluorophore. The optical configuration used for SPCE is similar to that used for SPR, except for our use of a silver film. However, we have recently detected SPCE using gold films of the type used in SPR. It appears that SPCE will become widely used for measurement of biomolecules binding to surfaces.

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Abbreviations

HbBv	bovine hemoglobin solution
IgG	immunoglobulin G
KR	Kretschmann configuration
OD	optical density
RET	resonance energy transfer
RK	reverse Kretschmann configuration
SPCE	surface plasmon-coupled emission
SPR	surface plasmon resonance

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Fig. 1.
Angular distribution of the 595-nm fluorescence emission of Rhodamine Red-X-labeled anti-rabbit antibodies bound to the rabbit IgG immobilized on the 50-nm silver mirror surface.

Fig. 2. Fluorescence spectra of the Rhodamine Red-X-labeled anti-rabbit antibodies bound to the immobilized rabbit IgG observed at 77° in RK-SPCE configuration (see Fig. 1); p and s refer to the orientation of the emission polarizer.

Fig. 3. Binding kinetics of the Rhodamine Red-X-labeled anti-rabbit antibodies bound to rabbit IgG immobilized on a 50-nm silver mirror surface observed with KR/SPCE configuration (top). Bottom: emission spectra measured after 60 min.

Fig. 4.

Emission spectra of the Rhodamine Red-X-labeled anti-rabbit antibodies bound to rabbit IgG immobilized on a 50-nm silver mirror surface in presence of a fluorescent background (anti-mouse antibodies labeled with Alexa Fluor 647) measured with different optical configurations.

Fig. 5.

Fluorescence spectra (SPCE) of the Rhodamine Red-X-labeled anti-rabbit antibodies bound to the rabbit IgG immobilized on a 50-nm silver mirror surface in absence (—) and presence (---) of highly absorbing background (bovine hemoglobin) observed with the KR/SPCE configuration.

Scheme 1.

Binding of anti-rabbit antibodies (labeled with Rhodamine Red-X) to rabbit IgG immobilized on the silver surface. Non-binding anti-mouse antibodies labeled with Alexa Fluor 647 remain in solution.

Scheme 2.

Experimental geometry for measurements of free space and SPCE emission with reverse RK and KR configurations.