Plastic Versus Glass Support for an Immunoassay on Metal-Coated Surfaces in Optically Dense Samples Utilizing Directional Surface Plasmon-Coupled Emission

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We compared plastic (polycarbonate) and high-quality glass support materials for gold-coated slides, when performing a model immunoassay against rabbit IgG using fluorescently labeled (AlexaFluor-647) anti-rabbit IgG, and detecting surface plasmon-coupled emission (SPCE) signals. Both, glass and plastic slides were simultaneously coated with a 48-nm layer of gold and protected with a 10-nm layer of silica. The maximum SPCE signal of AlexaFluor-647 was only two- to three-fold smaller on plastic slides than on glass slides. A small difference in the SPCE angles on glass ($\theta_F = 55°$) and plastic ($\theta_F = 52.5°$) slides was observed and can be explained with a slightly smaller refractive index of the plastic. We have not found any difference in the angle distribution (sharpness of the fluorescence signal at optimal SPCE angle) for the plastic slide compared to the glass slide. The kinetics of binding was monitored on the plastic slide as well as on the glass slide. Optically dense samples, a 4% red blood cell suspension and a 15% hemoglobin solution, are causing a reduction in the immunoassay SPCE signal by approximately 15% and three times, respectively, and the percentage of the reduction is the same for plastic and for glass slides. We believe that plastic substrates can be readily used in any SPCE assay, with only marginally lower total signal compared to high-quality glass slides.

KEY WORDS: Plastic slides; glass slides; fluorescence immunoassay; surface plasmon-coupled emission; gold film; background suppression.

INTRODUCTION

The low cost of plastic materials and the ease of material processing make them ideal candidates for many biotech applications where cost is critical, in particular, for performing surface assays on chips or in microfluidic channels [1–8]. Plastic chips can be made in a wider range of size, thickness, and function than glass chips [3]. The low production cost of plastic substrates allow for developing economical single-use device arrays, thus eliminating cleaning steps and avoiding sample-to-sample carry-over contamination [4,5]. One of the primary advantages of using plastic substrates for microfluidic systems is the ease with which devices can be fabricated in very large numbers [6]. Also, the surfaces of many plastic materials are compatible with biofluids and biomaterials, and well suited for applying complex surface chemistries [7–10].

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[6] ABBREVIATIONS: Ab, Antibody; Hb, Hemoglobin; IgG, Immunoglobulin G; KR, Kretschmann configuration; RBC, Red blood cell; SPCE, Surface plasmon-coupled emission; SPR, Surface plasmon resonance.

High-sensitivity fluorescence immunoassays are extensively used in diagnostics [11–15]. Several approaches have been suggested to improve sensitivity and to minimize the background signal caused by the sample matrix. These include kinetics detection [16], time-gated detection based on long-lived lanthanide fluorescence emission [17–19], and two-photon excitation [20,21]. Because of the high fluorescence background of physiological fluids, immunoassays are rarely carried out in whole blood, and in cases when they are, the procedure normally includes at least one washing step before the assay signal is measured [19,22–25].

In this report, we demonstrate the applicability of the surface plasmon-coupled emission (SPCE) based detection technique in connection with the use of plastic slides coated with a gold layer. Importantly, this method is being applied directly in optically dense samples mimicking whole blood, namely a 15% hemoglobin (Hb) solution, or a 4% red blood cell (RBC) suspension. This is possible due to an efficient collection of emitted fluorescence photons from a narrow 200–300 nm region near the assay surface. The method is based on the resonant coupling of excited fluorophores with electron oscillations in a thin metal film (typically, silver or gold) called surface plas-

mons. SPCE is closely related to surface plasmon resonance (SPR) [26–29]. One could consider SPCE as a reverse process to SPR, i.e., excited fluorophores near the metallic layer may induce surface plasmons in the metallic film. At certain conditions, these plasmons can radiate into the glass substrate at a sharply defined angle, and are almost completely polarized [26–30]. The interaction of excited dipoles with metal is a near-field resonance effect occurring without the emission of photons.

The excitation of surface-bound fluorophores is most effective with an attenuated total internal reflection set-up, called Kretschmann (KR) configuration, which has been described elsewhere [22–24]. When in a KR set-up the angle of the incident excitation beam, $\theta_I = \theta_{SP}$, where θ_{SP} is the specific angle of incidence that satisfies exact wave vector matching conditions, surface plasmons are excited, resulting in a sharp reflectivity decrease. The excited surface plasmons induce an evanescent field above the metal film surface. This evanescent field is strongly enhanced (up to 80-fold compared to the incident light intensity) by the resonance interaction [27], and extends up to 200–300 nm into the liquid sample. Hence the KR illumination results in a strong selective excitation in close proximity to the metal surface. The enhanced excitation region is
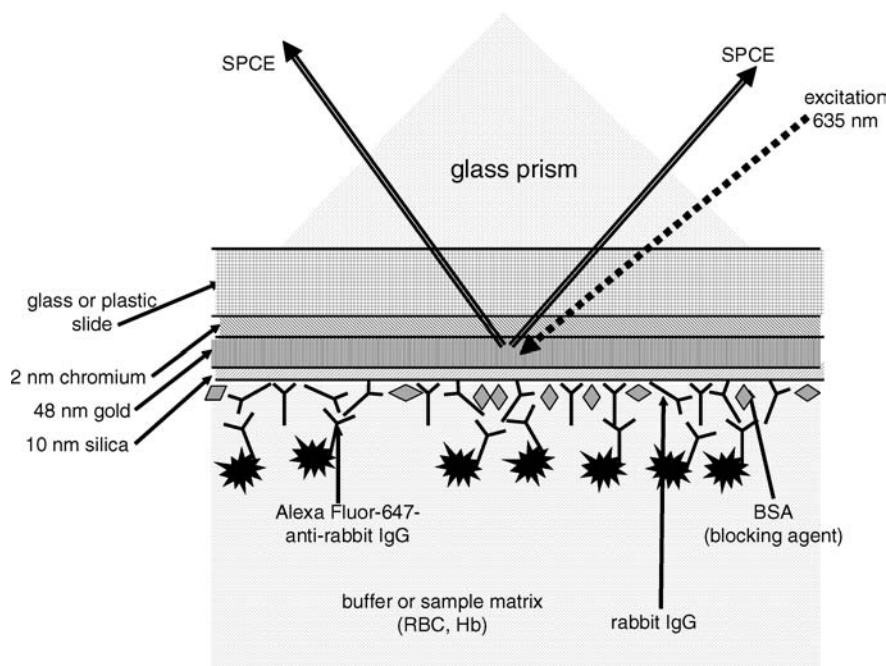
Fig. 1. SPCE immunoassay experimental scheme: Binding of anti-rabbit antibodies (labeled with AlexaFluor-647) to the antigen, rabbit IgG, immobilized on gold-coated plastic or glass slides, protected with a thin silica layer (the drawing is not to scale). The 635-nm beam excites the sample at the SPR angle θ_I (Kretschmann configuration, KR). The SPCE signal of AlexaFluor-647 is emitted at the SPCE angle θ_F.

confined to the evanescent-field layer, which effectively reduces the background from the sample volume matrix above.

A number of theoretical [26–29,31–32] and experimental [30, 31,33–43] papers on SPCE and its applications for ultra-sensitive detection have been published in the last few years. Recently, we introduced a model immunoassay [34,35,43] and a myoglobin immunoassay [36] on a thin silver metal surface utilizing SPCE.

In this work, we have tested the possibility of using plastic (polycarbonate) substrates for SPCE immunoassays in optically dense media by performing a model immunoassay against rabbit IgG, using fluorescently labeled anti-rabbit IgG (Fig. 1).

EXPERIMENTAL

Materials

Glass microscope slides (Corning, 3 in × 1 in) or plastic polycarbonate/Lexan slides (McMASTER-CARR, 3 in.×1 in.) were vapor-deposited with continuous 2-nm thick chromium, 48-nm thick gold, and 10-nm thick SiO_2 layers at the EMF Corporation (Ithaca, NY). Coated slides were manually pre-cut to halves (approximately 1.5 in.×1 in.) to better fit a demountable cuvette. Rabbit IgG, goat IgG, hemoglobin from bovine blood, and red blood cells (human AB, 4% suspension in phosphate-buffered saline) were obtained from Sigma, and AlexaFluor-647-anti-rabbit IgG conjugate (stock solution, 2 mg/mL, dye/IgG = 4.5 mol/mol) from Molecular Probes. Salts and buffer components (such as bovine serum albumin, Tween-20, and sucrose) were from Sigma-Aldrich.

Coating Slides with Antigen

Slides were covered with black tape containing rectangular (1 cm × 2 cm) holes to form wells on the surface of the slides (Fig. 2). Then slides were non-covalently coated with rabbit IgG: coating solution of IgG (dissolved to 40 μg/mL in sodium phosphate buffer, 50 mM, pH 7.4) was added to the slide (0.2 mL per well), and the slide was incubated for 1 hr at room temperature in a humid chamber. The slides were then rinsed with water, washing solution (0.05% Tween-20 in water), and again water. Blocking was performed by adding 0.25 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 room temperature for 1–2 hr (or overnight at +4°C) in the humid chamber. Then the slides
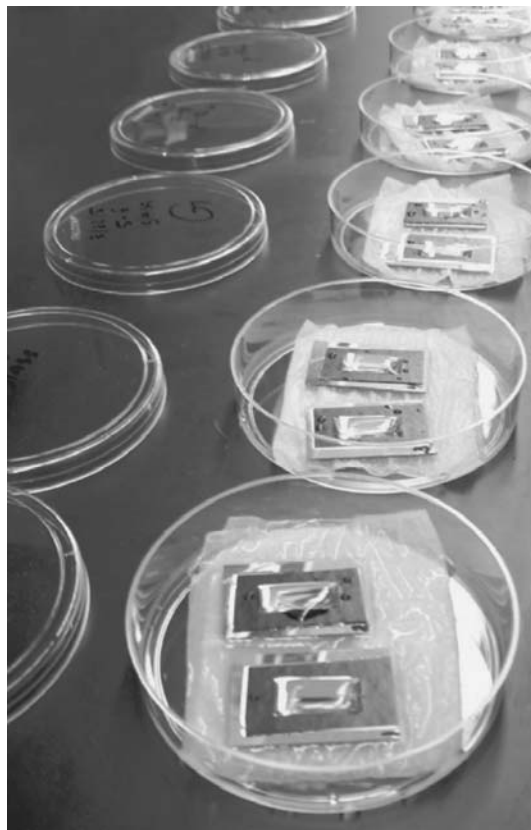
Fig. 2. Slides covered with black tape (forming wells) prior to the incubation in humid chambers.

were rinsed with water, washing solution (0.05% Tween-20 in water), and water, covered with blocking solution, and stored at +4°C until use.

End-Point SPCE Experiments

Dye-labeled AlexaFluor-647-anti-rabbit IgG (dye/IgG = 4.5 mol/mol; diluted to [IgG] = 10 μg/mL with blocking solution) was added to the slide (coated with antigen as described above, 0.2 mL/well) and incubated at room temperature in a humid chamber for 1 hr. Then the slide was rinsed with water, washing solution (0.05% Tween-20 in water), and water. Next, a 0.2 mm thick demountable quartz cuvette was mounted on the metallic side of the slide. About 40 μL of the blocking solution or a sample background solution (15% Hb or 4% RBC) was added inside the cuvette using a needle, and fluorescence measurements were performed in the Kretschmann or reverse Kretschmann optical configuration.

Kinetic Binding SPCE Experiments

A 0.2-mm thick demountable cuvette was mounted on the metallic side of the slide (coated with antigen as described above). About 40 μL of the AlexaFluor-647-anti-rabbit IgG (dye/Ab = 4.5 mol/mol; diluted to [IgG] = 10 μg/mL with blocking solution) was added inside the cuvette using a needle. The kinetics was immediately monitored at room temperature (approximately 20°C).

Spectroscopic Measurements

Absorption spectra were measured using a Hewlett Packard model 8543 spectrophotometer and 0.2-mm path length cuvettes. The recorded spectra showed that a 0.2-mm thick layer of 4% RBC suspension had an optical density of about 1.3–1.4, and a 15% Hb solution in blocking buffer had an optical density of about 0.7–0.8 at 670 nm, which is the emission maximum of the bound labeled antibodies.

Fluorescence measurements on microscope slides were performed using index-matching fluid to attach the slide to a triangle prism made of SF-11 glass (with a refractive index of 1.785) and positioned on a precision rotary stage equipped with a fiber optics mount on a 15-cm long arm [30]. This configuration allowed for fluorescence observation at any angle relative to the incident angle. For collection of the angle-dependent emission intensity, a 200-μm air slit was placed on the fiber input. The output of the fiber was connected to an SLM model 8000 spectrofluorometer (SLM Instruments, IL, USA). The fluorescence excitation was generated using a small solid-state red laser diode module (635 nm, powered by two 1.5 V AA batteries, maximum output 2 mW) as commonly used in commercial laser pointers.

RESULTS AND DISCUSSION

Our experimental scheme for the SPCE immunoassay is shown in Fig. 1. The sample was held in the thin cuvette mounted on the slide (plastic or glass), coated with a gold film. AlexaFluor-647-labeled antibody was bound near the gold surface to the surface-immobilized antigen. The sample with assembled cuvette was illuminated in the KR configuration, as shown in Fig. 1. SPCE was observed on the prism side of the sample, at the plasmon angle through a long-pass filter. The intensity observed through the prism was sharply directed near the θ_F angle, approximately 55° for a glass slide, and approximately 52.5° for a plastic slide (Fig. 3). These values are close
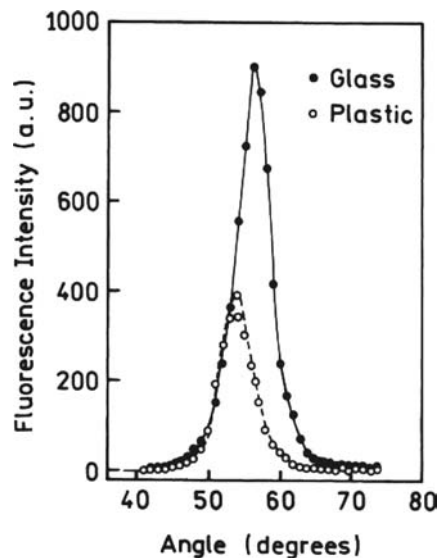

Fig. 3. Angular distribution of the 670-nm fluorescence emission of AlexaFluor-647-labeled anti-rabbit antibodies bound to the rabbit IgG immobilized on the gold-coated plastic (○) or glass (●) slide surface.

to those calculated from minimum reflectance for the p-polarized plasmon mode for glass and plastic materials (Fig. 4).

In the end-point experiments, AlexaFluor-647-labeled Ab was first bound to immobilized rabbit IgG near the silver surface. Then, the excess of non-bound
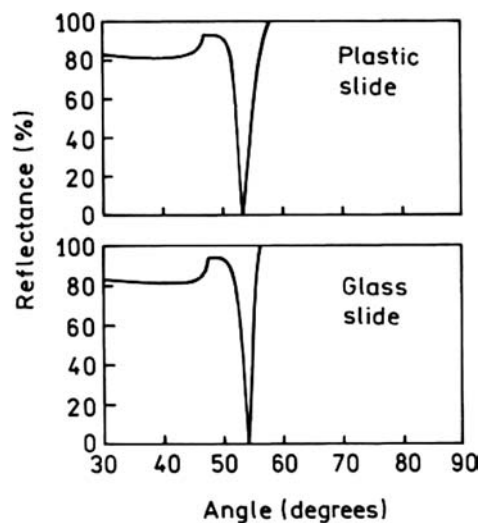

Fig. 4. Calculated reflectivities of the six-layer system (SF-11 prizm, substrate, gold layer, silica layer, protein layer, and buffer) as described in Fig. 1, for glass and for plastic substrates. The dielectric constant for gold at 670 nm was $\varepsilon_m^{670} = -13.14 + 1.04i$; the refractive indices of glass and plastic were 1.51 and 1.43, respectively.

antibodies was washed out, and a sample matrix was added (4% RBC, or 15% Hb, or just blocking solution for comparison), and the fluorescence signal and spectrum were monitored. We used these sample matrixes as models for the clinical (whole blood) samples. The absorption spectra showed that a 0.2-mm thick layer of 4% RBC suspension had an optical density of about 1.3–1.4, and a 15% Hb solution in blocking buffer had an optical density of about 0.7–0.8 at 670 nm, the emission maximum of the bound labeled antibodies. The emission spectrum of the SPCE (in KR configuration) was characteristic for the AlexaFluor-647 probe and was not corrupted by scattered light at the excitation wavelength for all tested sample matrixes (Fig. 5). Our study on the effect of a highly absorbing sample matrix on the recorded SPCE signal for gold-coated glass and for plastic slides (Fig. 5) showed that the signal is decreased only by 10% to less than 20% in the 4% RBC suspension, and approximately 2.5–3-times in the 15% Hb solution, compared to the buffer medium (blocking solution). These results are very similar to the results we had obtained earlier on silver-coated glass slides using a17% Hb solution ([36], describing a myoglobin immunoassay using Rhodamine Red-X-labeled antibodies) or whole blood ([43], describing a model immunoassay using AlexaFluor-647-labeled antibodies) as a sample matrix.
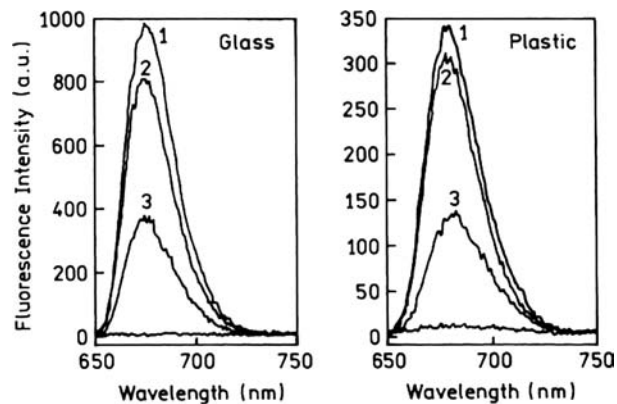
We were puzzled by the fact that the RBC suspension reduces the SPCE signal only by 10–20%, while the Hb solution (with an optical density almost twice lower than in the RBC suspension) reduces the SPCE signal 2.5–3-fold. We speculate that this is a size-related effect of an absorber. Small Hb molecules would fit easily into the 200–300 nm deep evanescent-field surface plasmon re-

gion. In contrast, in the case of RBCs with their relatively large size of several microns, only a small fraction of their volume is located within the evanescent-field region.
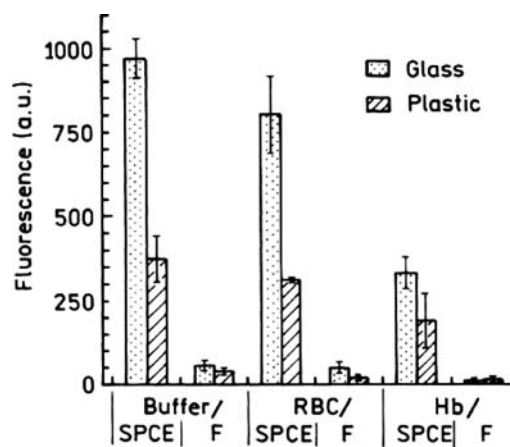
We noticed that in the experiments, when comparing the signal from the glass slide to the signal from the plastic slide under similar conditions (same optical configuration, same sample matrix), the attenuation is twice as high in the case of the plastic devices (Figs. 5–7). We believe that this is related to the slightly lower refractive index of plastic ($n = 1.43$) than glass (1.51), and to the quality of the substrate surface.

Figure 6 provides a visual comparison of fluorescence signals from glass and from plastic slides for SPCE and for free-space (F) emission. Although the SPCE signal from the plastic slide is only about one-half of the signal obtained on the glass slide, the signal ratio SPCE/F is almost constant. We conclude, however, that the SPCE signal from plastic slides is sufficiently strong so that it can be used not only for performing steady-state fluorescence measurements in optically dense samples (Fig. 6), but also for studying the kinetics of binding.

We have been able to monitor the binding kinetics to the surface-immobilized antigen directly in the sample matrix by measuring the SPCE signal of the AlexaFluor-647-labeled antibodies in RBC suspensions and in Hb solutions for both types of slides (glass and plastic). By testing both types of slides with blocking solution, with RBC suspension, and with HB solution, we observed the

Fig. 6. Fluorescence signal measured at different optical configurations in various background solutions (buffer vs. 15% Hb and RBC): Comparison of the glass (*dotted bars*) and plastic (*cross-hatched bars*) slide support. The fluorescence signal was detected in SPCE configuration (see description in text), with excitation and detection from the prism side; the free-space (F) signal is detected with excitation from the sample side and detection from the prism side. Error bars represent one SD ($n = 4$, except for RBCs on plastic, where $n = 3$).

Fig. 5. SPCE spectra of the sample on glass (*left*) or plastic (*right*) support in buffer (1) and in presence of the absorbing background: RBC (2) or 15% Hb solution (3). Data represent an average of two fluorescence spectra, taken on different days, for each sample matrix.
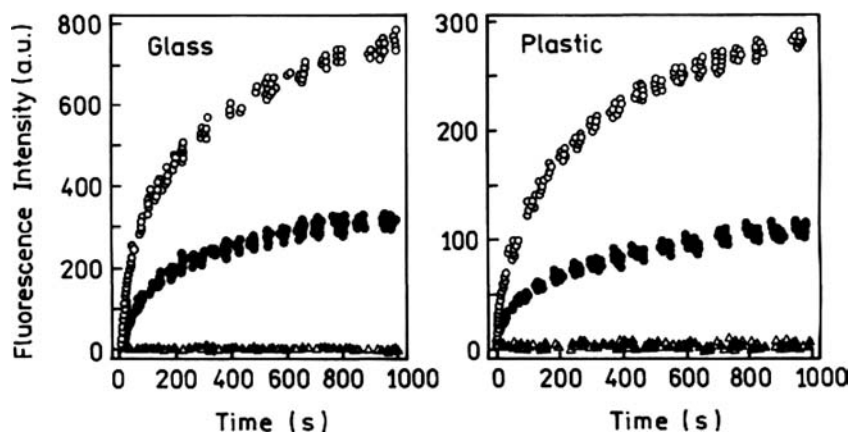
Fig. 7. SPCE kinetics of binding of the labeled anti-rabbit IgG to the rabbit IgG immobilized on the slide with glass (*left*) or plastic (*right*) support: (○) in blocking buffer; (●) in a 15% Hb solution. Triangles ((△) in buffer; (▲) in the 15% Hb solution) represent corresponding control experiments (non-specific binding to the immobilized goat IgG). The data represents an average of two kinetics (taken on different days) for each sample matrix.

same relative attenuation of the signal on plastic versus glass when studying the kinetics of binding (Fig. 7, circles). The kinetic curves shown in Fig. 7 illustrate also the relationship between specific binding and non-specific binding of the labeled anti-rabbit Abs to the wrong antigen (goat IgG) under identical conditions (Fig. 7, triangles). Non-specific binding results in an SPCE signal of less than 10 arbitrary units, which corresponds approximately to the instrument noise floor.

CONCLUSIONS

We have successfully demonstrated that immunoassays can be performed in optically dense samples such as whole blood, using directional surface plasmon-coupled emission of fluorescence on metal-coated surfaces of plastic substrates. Compared to substrates made out of high-quality glass, the SPCE signal on plastic substrates is roughly two times lower, but still strong enough to allow for steady-state as well as for kinetic measurements. The low production cost of plastic substrates would allow for developing economic disposable assay devices and mass production. Moreover, the surfaces of many plastic materials are compatible with biofluids and biomaterials, and well suited for applying complex surface chemistries.

ACKNOWLEDGMENTS

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