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Sensitive bioimaging in microfluidic channels on the plasmonic substrate: Application of an enhanced fluorescence based on the reverse coupling mode

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ABSTRACT

Grating-coupled surface plasmon-field enhanced fluorescence (GC-SPF) was applied to biosensing. Although the greatest enhancement of GC-SPF on our plasmonic chips compared with that on a glass slide was found to be 40 times, this was due to the enhanced excitation field. Therefore, grating-coupled fluorescence using a reverse coupling mode is here explored to achieve further 4–5 times enhancement. As a result of using both the excitation field enhanced by grating-coupled surface plasmon resonance and the directional emission enhanced by reverse coupling mode, an increase in fluorescence of more than 110 times compared with that on the glass slide was recorded. The reverse coupling mode was also applied to the sensitive fluorescence microscopic imaging of Cy5-streptavidin (Cy5-SA) in microfluidic channels on a two dimensional nanohole array substrate. We performed a Cy5-SA concentration series analysis in which the plasmonic substrate demonstrated 26.3× enhancement of sensitivity and a limit of detection (LOD) of *ca.* 100 pM, which is at least one order of magnitude lower than in glass slides with identical surface chemistry. This plasmonic nanostructure will be invaluable for colorimetric detection in applications such as microfluidic enzyme-linked immune-sorbent assay (ELISA) device, and portable microarray biosensing, because the optical setup can be simplified.

1. Introduction

In grating-coupled surface plasmon resonance (GC-SPR), which is known to be a kind of propagated plasmons, the incident light is introduced to the interface by trapping using a metallic periodic structure instead of a prism [1–5]. The advantage of GC-SPR is that the resonance angle can be controlled by the pitch of the grating. On the other hand, in the Kretschmann configuration of prism-coupled surface plasmon resonance (PC-SPR), even if a prism with high refractive index (e.g. 1.85) and light with a longer wavelength (e.g. 633 nm) could be used, the resonance angle would be above 60° for the water interface [6,7]. The application of PC-surface plasmon-field enhanced fluorescence (SPF) to bio-sensing is complex in operating and constructing optical stuffs for illumination and difficult in using a fluorescence microscope. The lower incident angle in GC-SPR without prism facilitates operation and simplifies the optical setup. Bio-sensors using the fluorescence excited by the enhanced electric fields of GC-SPR, which is known as grating-coupled surface plasmon-field enhanced fluorescence (GC-SPF), are being developed as an application and a number of relevant reports have already been published [8–10]. We have applied GC-SPF specifically to fluorescence microscopic imaging and biosensor-based fluorescence detection [11–14]. In the former application, fluorescence images of cells were more than 20 times enhanced compared with images taken on glass slides [11]. In the latter application, 40 times enhanced fluorescence was detected in a biochip with an optimized grating structure in which the pitch, groove depth, and duty ratio (ratio of convex part) after silver coating were 400 nm, 20 nm, and 0.5, respectively; the surface profile was trapezoid [12–14].

Meanwhile, the reverse coupling mode recently described with the term surface plasmon-coupled emission (SPCE) has been studied in the reverse Kretschmann configuration of PC-SPR [9,15–17]. Fluorescence from molecules may excite SPP modes at the fluorescent dye/metal interface, and these SPP modes may in turn decay to produce light in the prism. Such an SPCE technique has been performed on many structures but seldom on a grating structure. Chiu et al. [9] studied the effect of the reverse coupling mode on the active emission of a grating with organic semiconductor material, Alq_3, on the surface. Feng et al. [18] also reported highly directional emission by GC-SPR tunneling from electroluminescence in organic light-emitting devices. In the present study, on the other hand, the application of the reverse coupling mode to the emission from fluorescent dye-labeled proteins was investigated in biochips with

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silver-coated grating, i.e., in plasmonic chips. The use of reverse coupling mode can simply make the bio-sensing in the lower target concentration possible. We found that the use of the both the enhanced excitation field produced by SPP resonance and the directional emission from the reverse coupling mode provided a very significant fluorescence enhancement in the biochip. Therefore, the reverse coupling mode can be applied to fluorescence microscopic imaging in microfluidics channels.

Microfluidic lab-on-chip devices and micro-total-analysis systems have been widely investigated to advance and simplify complex biosensing applications on small chips [19,20]. In the microfluidic nanohole array, proteins and DNA were sensitively detected by the enhanced surface plasmon resonance signals without fluorescent probe [21,22]. However, the sensitivity is not superior to the fluorescence methods. In colorimetric measurement, which is one of the most popular detection methods using microfluidics techniques [23], fluorescence-based imaging detection has produced detection limits comparable to those of enzyme-linked immune-sorbent assay (ELISA) [24–26]. In this study, fluorescent-labeled marker proteins were detected in the flow system with microfluidic channel device, differing from the sink system used in our previous studies [14]. Flow system makes an evaluation of affinity possible in a bioassay and is a useful technique. The combination of microscope, microfluidic channel, and plasmonic chip achieved highly sensitive fluorescence detection under an easy operation and a simple setup. Various optically passive substrates, such as glass [27], plastic [28,29], PDMS [25,30], and UV-curable epoxy resin [31], have been used in microfluidic devices. Chemical modification of the surfaces is one way to improve the performance of these biosensing devices by eliminating non-specific binding while enhancing biological activity and stability [32,33]. Enhancing the fluorescence of the fluorophores near the surface has proved to be another effective way of improving the performance of fluorescence biosensing systems [34]. For example, photonic crystal surfaces consisting of periodic TiO_2 have been used to enhance the fluorescence detection of protein microarrays [35]. By evaluating the bioassay in a concentration series on plasmonic and glass substrates, we demonstrated the successful application of these plasmonic substrates in a microfluidic channel device.

2. Experimental

2.1. Grating substrate

Grating structures were constructed on two sheets of SiO_2 substrates using a previously reported two-laser-beam interference method followed by dry etching [36]. Briefly, the photoresist pattern was formed by exposing to the interference beam of a He–Cd laser (Kimmon Electric, 1K3501R-G) with 325 nm wavelength, after which the SiO_2 surface was etched by dry etching (ULVAC, NLD-500). The two dimensional (2D) substrate was also fabricated in two identical exposure steps, the substrate being rotated by a 90° angle approximately normal to its surface between exposures. The residual photoresist was then removed by acetone washing and then oxygen plasma treatment. (Yamato, RFG-500A). The slide glass plates (used for control experiment) and grating substrates were cleaned by sonicating in a 1% Hellmanex (Hellma, Müllheim, Germany) solution and rinsing extensively with fresh Milli-Q water, after which they were dried completely under air flow. The fabricated 1D gratings and 2D grating were measured by scanning probe microscopy (SPM). The pitches of the two 1D substrates were 402 and 410 nm (error in measurement by SPM: ±10 nm), the groove depths were found to be 25, and 22 nm (error in SPM measurement: ±3 nm), while the duty ratios before coating (ratio of convex part to the pitch) differed, being 0.71 and 0.56 (error in SPM measurement: ±0.03), for gratings Nos. 1 and 2, respectively. The pitch of the 2D nanohole array was 400 ± 10 nm, the groove depth and the duty ratio were 31 ± 3 nm and 0.33 ± 0.03.

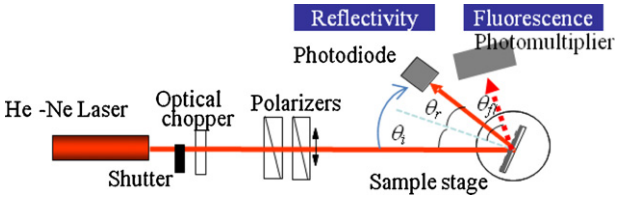

Fig. 1. SPR-SPFS setup.

Thin metal layers covered with a SiO_2 overlayer (with an adhesion layer of Cr) were prepared on both substrates via the following process. First, a Cr layer of less than 1 nm thickness was deposited using an rf sputter setup (Rikensya, specially made) in an Ar gas flow. A 100-nm-thick silver layer and 200-nm-thick silver layer was then deposited on the Cr layer and another Cr layer was deposited for 1D and 2D substrates, respectively, without opening the chamber. Finally, a 20-nm thick SiO_2 layer was deposited on the Cr layer for both substrates. A 20-nm thick SiO_2 layer efficiently suppresses (quenches) energy transfer to the metal layer from the fluorescence excited by the GC-SPR field [8].

The coated substrates were modified with 3-aminopropyltriethoxysilane (APTES, Sigma–Aldrich) aqueous solution (1 vol.%) for 1 h. After reaction, the APTES solution was removed and the substrates rinsed by immersion in Milli-Q water (18.2 MΩ) and ethanol and dried. For detection of labeled proteins, biotin-poly(ethylene glycol)-carbonate-NHS ester [NOF SUNBRIGHT BI-050TS] prepared in aqueous solution (2 mM) was poured onto the surface of the substrates, which were rinsed with Milli-Q water at 1 h after the reaction between the NHS group and the amino group of the substrate surface.

2.2. Reflectivity and fluorescence measurement

A He–Ne laser beam of 632.8 nm wavelength passes through an optical chopper (used also as the reference for the lock-in amplifier) and two polarizers for intensity and polarization control as shown in Fig. 1. Using a $\theta - 2\theta$ goniometer, the light reflected at the substrate is monitored against the incident angle (θ_i) with a photodiode automatically rotating at $\theta_i + \theta_r$ ($\theta_i = \theta_r$). The emission [after passing through a narrow band interference filter, $\lambda = 670 \pm 5$ nm, and notch filter (stopline, Semlock, USA)] is monitored with a photomultiplier mounted on the goniometer stage in the following two ways: incident angle (θ_i)-scanning fluorescence measurement with a photomultiplier fixed at 55° for the incident beam-line; and θ_{fl}-scanning fluorescence measurement under the fixed incident angle θ_i of resonance angle, 30° and 45°. In the fluorescence detection under a chip rotation for axis normal to surface, non-polarized light was incident using a depolarizer.

2.3. Samples

A labeled protein [streptavidin (SA) labeled with Cy5 (Amersham)] was prepared at a concentration of 100 nM with phosphate buffered saline solution for measurement of angle-scanning reflectivity and fluorescence intensity. In order to precisely evaluate the fluorescence enhancement factor on the plasmonic chip compared with the fluorescence intensity measured on the glass slide, the concentration of Cy5-SA of 100 nM was appropriate, because the fluorescence intensity was not precisely measured on the glass slides for 10 nM-Cy5-SA. A cover glass was attached to the top of all substrates and a 20 μL solution was injected into the substrate by