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Application of 300× Enhanced Fluorescence on a Plasmonic Chip Modified with a Bispecific Antibody to a Sensitive Immunosensor

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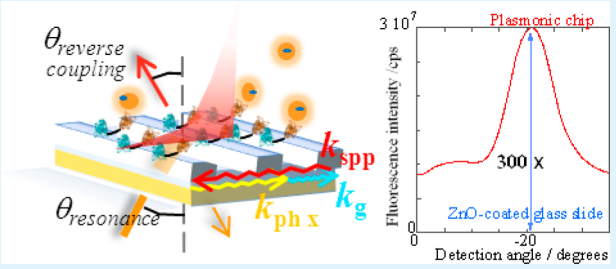
Supporting Information

ABSTRACT: The grating substrate covered with a metal layer, a plasmonic chip, and a bispecific antibody can play a key role in the sensitive detection of a marker protein with an immunosensor, because of the provision of an enhanced fluorescence signal and the preparation of a sensor surface densely modified with capture antibody, respectively. In this study, one of the tumor markers, a soluble epidermal growth factor receptor (sEGFR), was selected as the target to be detected. The ZnO- and silver-coated plasmonic chip with precise regularity and the appropriate duty ratio in the periodic structure further enhanced the fluorescence intensity. As for sensor surface modification with capture antibody, a bispecific antibody (anti-sEGFR and anti-ZnO antibody), the concentrated bispecific antibody solution was found to nonlinearly form a surface densely immobilized with antibody, because the binding process of a bispecific antibody to the ZnO surface can be a competitive process with adsorption of phosphate. As a result, the interface on the plasmonic chip provided a 300× enhanced fluorescence signal compared with that on a ZnO-coated glass slide, and therefore sEGFR was found to be quantitatively detected in a wide concentration range from 10 nM to 700 fM on our plasmonic surface.



KEYWORDS: *plasmonic chip, bispecific antibody, immunosensor, enhanced fluorescence, grating, zinc oxide*

INTRODUCTION

The surface plasmon resonance (SPR) method is well-known as a powerful nonlabel tool for an immunosensor, but the SPR field is also attractive as an enhancer for a fluorescence bioprobe. The grating-coupled SPR (GC-SPR) is one of the propagated SPR forms that provides direct coupling between surface plasmon polaritons and radiation modes,[1,2] and the resonance condition was described using the resonance angle (θ_r) and wavevectors of surface plasmon polaritons (k_{spp}), incident light (k_{ph}), and grating (k_g) as

$$k_{spp} = k_{ph} \sin \theta_r + mk_g \qquad (m = \pm 1,\ 2,\ 3,\ \ldots) \tag{1}$$

The electric-field intensity of the SPR field was known to be several hundred times enhanced. The enhancement factor of the electric-field intensity (E_f) in the grating with a sinusoidally modulated height profile can be described[1] as

$$E_f = 2|\varepsilon_m'|^2 \cos\theta(1 - R)/(\varepsilon_m''(|\varepsilon_m'| - 1)^{1/2}) \tag{2}$$

in which ε_m' and ε_m'' are the real part and imaginary parts, respectively, of a silver's complex dielectric constant and θ and R are the incident angle and reflectivity. A greater fluorescence enhancement is therefore expected on a grating surface with a small incident angle and low reflectivity (=efficient coupling with incident light). Therefore, when the GC-SPR field is utilized as the excitation field for fluorescent molecules, it can nonlinearly intensify the fluorescence of fluorescent molecules on metal-coated chips with a grating structure, i.e., so-called plasmonic chips,[3−6] in which the pitch has the wavelength scale of UV−visible light. We have developed a marker detection system using a plasmonic chip.[3−6] The plasmonic chip needs precise fabrication with the required pitch, groove depth, and duty ratio (ratio of the convex part to the pitch), and it makes possible effective coupling with incident light and can contribute to sensitive marker detection. To construct a highly sensitive grating-coupled surface plasmon field-enhanced fluorescence (GC-SPF) detection system, the plasmonic chips should be designed not only to optimize the chip structure but also to efficiently immobilize capture antibodies on the chip surface. Therefore, it is important that the chip surface homogeneously and densely immobilized with capture antibody can be briefly achieved.[7] Recently, several peptides and antibodies with affinity for nonbiological materials were identified using a combinatorial library approach,[8−13] and they spontaneously bind onto a specific inorganic material surface. By fusion of the peptides and antibody fragments to the proteins, the fused proteins can be directly immobilized with

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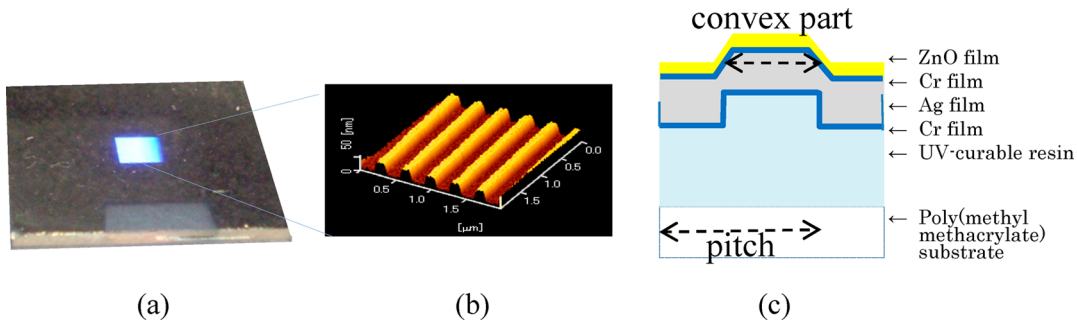

Figure 1. Plasmonic chip: (a) photograph of the plasmonic chip, with the blue area corresponding to a part of the periodic structure; (b) scanning probe microscopic image of the grating coated with silver and ZnO films (the full scales of the *X* and *Z* axes were 2 μm and 50 nm, respectively); (c) cross-sectional scheme of the grating chip.

desired orientations without the need for surface fabrication of the substrate or complicated conjugation processes.[8,10,14−16] In previous studies, we generated antibody fragments that densely bound onto a ZnO surface with high affinity,[8] and we designed chimeric antibodies with a bispecific affinity for a ZnO surface and green-fluorescent protein (GFP).[7] The ZnO surface is one of the attractive materials in the various fields including optical and electronic devices, and its transparency and high refractive index were important for the optical sensor chip as our plasmonic chip. The bispecific antibodies were rapidly applied to a ZnO-coated plasmonic chip, and 10 pM GFP was sensitively detected within 10 min under the simple operation.[7] The fluorescence intensity observed on the plasmonic chip was 100 times larger than that on the ZnO-coated glass slide.

In the present study, in order to improve the fluorescence enhancement and the limit of detection (LOD) of a marker compared with our previous study for GFP detection, the plasmonic chip with more precise regularity and the appropriate duty ratio[4] in the periodic structure as efficiently coupled with the incident light was fabricated and the bispecific antibody was more densely immobilized onto the ZnO−plasmonic chip. One of the tumor markers, epidermal growth factor receptor (EGFR), was selected as a detection marker, and the plasmonic chip was immobilized with a recombinant antibody having a bispecific affinity for an antigen protein and a specific inorganic material surface.[8−10] EGFR is widely expressed on various solid malignant tumors, including bile duct cancer,[17,18] and the soluble EGFR (sEGFR) isoform (extracellular fragments of EGFR), which is present in serum, is expected to be a theranostic cancer biomarker for breast, lung, and ovarian cancer.[19] For practice of the detection system in clinics, highly sensitive detection, a wide detection range, good reproducibility, rapid and simple operation, and a small instrument are required, and they may be solved by our plasmonic chip.

EXPERIMENTAL SECTION

Fabrication of a Plasmonic Chip. To fabricate the plasmonic chip, a UV-curable resin (PAK-02-A, Toyo Gosei) was dropped onto a poly(methyl methacrylate) substrate and exposed to UV light from the side of a mold. The grating area was 4 × 4 mm^2, and the replica with a precise and homogeneous periodic structure was supplied using the mold fabricated by electron-beam (EB) lithography (NTT-AT, Japan). A thin silver layer covered with a ZnO overlayer (with an adhesion layer of Cr) was prepared on the substrates. First, a Cr layer with a thickness <1 nm was deposited using an radio-frequency sputter setup (Cryovac, specially made). Then, the thickness of the deposited silver layer was controlled at 35 ± 3 nm, and chromium sputtering onto the silver layer was repeated without opening the chamber. Finally, a (17 ± 2)-nm-thick ZnO layer was deposited onto the chromium layer. The distance from the metal surface was controlled with the ZnO-layer thickness, and the appropriate film thickness of ZnO with a high refractive index was considered to be shorter than the film thickness (=20 nm) of the SiO_2 layer used previously.[20,21] Glass slides coated with a (17 ± 2)-nm-thick ZnO film (with an adhesion layer of chromium) were also prepared and used as references. The periodicity of the plasmonic chip used in this study, groove depth, and duty ratio (ratio of the convex part to the pitch) were 350 ± 10 nm, 33 ± 3 nm, and 0.50 ± 0.03, respectively.

Samples for Immunoassay. A bispecific recombinant antibody composed of anti-EGFR and anti-ZnO VHH domains (M_w = 31562 g mol^{-1}) was prepared according to a previous report. Extracellular domains of human EGFRs (sEGFR; PeproTech)[8] were labeled with cy5 using a cy5-labeling kit (GE Healthcare). The labeling ratio of the cy5-labeled EGFR was evaluated at 4 ± 0.5. The bispecific antibody was prepared at 1−20 μM in a phosphate-buffered saline solution [10 mM phosphate (pH 7.5)/200 mM NaCl]. cy5-sEGFR solutions were prepared at concentrations ranging from 30 nM to 700 fM. A cover glass was attached to the top of all substrates, and the space between the chip and the cover glass was filled with an 8 μL sample solution using a pipet. The fluid thickness was thin, about 50 μm. The bispecific antibody can bind sufficiently to the ZnO layer on the plasmonic chip within 5 min. For rinsing, 10 × 2 μL of a phosphate-buffered saline solution was injected using a pipet. Detergent (Tween 20, 0.05%) was added to the cy5-sEGFR and buffered saline solutions, for rinsing and sample dilution. A labeled sEGFR solution was poured onto the chip surface, and cy5-sEGFR captured by the antibody was detected after rinsing, following an incubation time of 10 min. For the measurement of nonspecific adsorption, cy5-labeled bovine serum albumin (BSA; labeling ratio = 5.2) was prepared by the same labeling kit at concentrations of 25 nM.

Optical Measurement.[7,24] A helium−neon laser beam (wavelength, 632.8 nm) was used as an irradiation source. The diameter of the irradiation spot was about 1 mm. Using a θ−2θ goniometer, the substrate was set on the goniometer as the grating vector parallel to the incident plane, and the light reflected by the substrate under irradiation from the rear panel was monitored against incident angles (θ_i) of 5−35° with a silicon photodiode placed on another stage that rotated at 2θ. For fluorescence detection, a photomultiplier tube (PMT, specially made) was used instead of a photodiode. A short-wavelength light-cut filter (SC660, Sigma Koki), a narrow-band interference filter (λ = 670 ± 5 nm, Sigma Koki), and a notch filter (Stopline, NF03-633E, Semrock) were inserted in the front of the PMT. The emission was monitored against the detection angles (θ_{fl}) from 0° to 35° for the direction normal to the substrate when the incident angle was fixed at the resonance angle, 12°. All measurements were performed at room temperature (22−23 °C).

RESULTS AND DISCUSSION

Fluorescence Enhancement on the Plasmonic Chip due to the Excited Field Enhanced by the SPR Field and

Reverse Coupling. The grating replica with a 350-nm pitch was fabricated by UV−nanoimprint lithography (UV−NIL) and successively coated with chromium, silver, chromium, and ZnO films, as shown in Figure 1a. The topography of the ZnO/silver grating chip was measured by scanning probe microscopy (Figure 1b). The pitch of the grating and groove depth were 350 ± 10 and 33 ± 3 nm, respectively.

As a result of the reflectivity measured against the incident angle, the GC-SPR angle was observed at 12°, as shown in Figure 2a. The electric field of light irradiated at the resonance

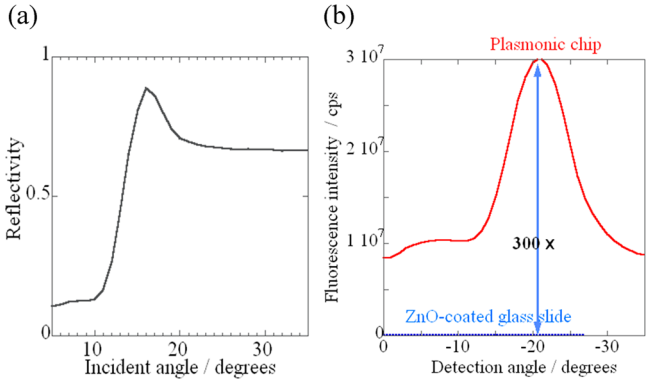

Figure 2. (a) Reflectivity measured against the incident angle and (b) fluorescence intensity of cy5-sEGFR measured against the detection angle under the incident angle fixed to the resonance angle.

angle was enhanced because of the SPR field, and it was applied to the excited field for the fluorescent molecules bound to the chip. Therefore, the fluorescence measured under the resonance condition was enhanced around 50 times. Further, the fluorescence of 10 nM cy5-sEGFR was monitored against the detection angle defined for the direction normal to the substrate. A fluorescence peak was found at a detection angle of 23°, assigned to the reverse coupling,[22−24] which is recoupled between plasmon polaritons and fluorescence. The reverse-coupling angle of 23° was predicted by the coupling condition described in eq 1 under a wavelength of 670 nm, and it was coincident with an experimental value. The fluorescence enhancement measured under the reverse coupling was 300 times compared with 1×10^5 cps, measured on the ZnO-coated glass for 10 nM cy5-sEGFR (Figure 2b). Application of the reverse-coupling mode and of a rear-irradiation system is recommended for the detection of cy5-sEGFR sensitively and surface-selectively.[24]

Highly Dense Immobilization of anAnti-ZnO × sEGFR Bispecific Antibody. A bispecific antibody with an affinity for the extracellular domain of EGFR and for ZnO surfaces was prepared as a sensitive strategy for detecting sEGFR. The single variable domain of the heavy chain of a camel antibody (VHH) with a high affinity for ZnO surfaces (dissociation constant, 9 nM), called 4F2,[8] fused to the C-terminus of an anti-EGFR VHH (la1)[25] via a lama IgG2 upper hinge linker (EPKIPQPQPKPQPQPQPQPQPKPQ PKP EP; Figure 3).

At the point of detection sensitivity, the density of the antibody immobilized to the chip surface is dominated. Therefore, because the density of the anti-sEGFR × ZnO bispecific antibody immobilized on the chip can be higher, the solution of an anti-sEGFR × ZnO bispecific antibody was prepared at higher concentration, 1−20 μM. After the bispecific antibody solution was incubated on the chip surface for 10 min

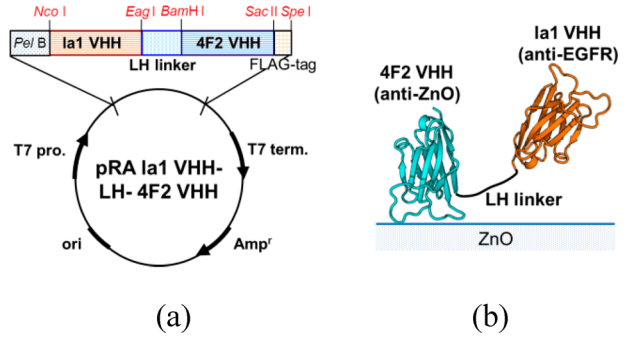

Figure 3. Vector map (a) and structural image (b) of the bispecific antibody with an affinity for EGFR and ZnO.

and rinsed with a phosphate-buffered saline solution, 10nM cy5-sEGFR was injected. After a further 10 min of incubation of antigen, the chip was rinsed, and then the fluorescence intensity was measured. The fluorescence intensity for 10 nM cy5-sEGFR was found to strongly depend on the concentration of the bispecific antibody solution injected as shown in Figure 4a. As the antibody concentration increased from 1 to 20 μM, the fluorescence intensity of cy5-sEGFR surprisingly increased by 4 orders, i.e., from 5×10^3 to 3×10^7 cps. The binding process of an anti-sEGFR × ZnO bispecific antibody to the ZnO surface can be a competitive process with adsorption of phosphate (see the Supporting Information); therefore, the concentrated bispecific antibody solution is considered to nonlinearly provide a dense immobilization of the antibody and capture more molecules of cy5-EGFR. As shown in Figure 4b, in the plasmonic chip prepared at 1 μM bispecific antibody, the detection of EGFR was possible at several nanomolars ($=10^{-9}$ M). When a bispecific antibody solution was prepared at 4.3 μM, EGFR was detected at tens of picomolars ($=10^{-11}$ M). At a concentrated solution of 20 μM, EGFR was detected at hundreds of femtomolars ($=10^{-13}$ M).

In our previous study,[7] the LOD of GFP was 10 pM, in which the anti-GFP × ZnO bispecific antibody was immobilized to the chip in a 1 μM solution. In this study, the anti-EGFR × ZnO bispecific antibody prepared at 20 μM contributed to the improvement of the LOD of a marker. Not only the higher density of anti-sEGFR × ZnO but also the precise regularity in the periodic structure and the favorable duty ratio provided a superior LOD compared with that in our previous study. The plasmonic chip fabricated by EB lithography was improved compared with our homemade chip. Further, the duty ratio of the former (this study) close to 0.5 was better than that of the latter (the previous study) at the point of fluorescence enhancement.[4]

LOD and Detectable Concentration Range in sEGFR Detection with a Plasmonic Chip. The highly concentrated bispecific antibody solution was expected to provide improvement in the LOD because of the dense immobilization of the antibody. However, the LOD depends on the performance of the total optical system including a PMT, a light source, and filters. In this study, the significant photon-counting value for the plasmonic chip providing the angular-dependent fluorescence was 5×10^3 cps at the resonance angle, and therefore the plasmonic chip is expected to evaluate the low concentration of EGFR. The LOD of tumor-marker detection with a plasmonic chip is as shown in Figure 5. The fluorescence intensities plotted in Figure 5 were obtained from the fluorescence peak measured against the detection angle of

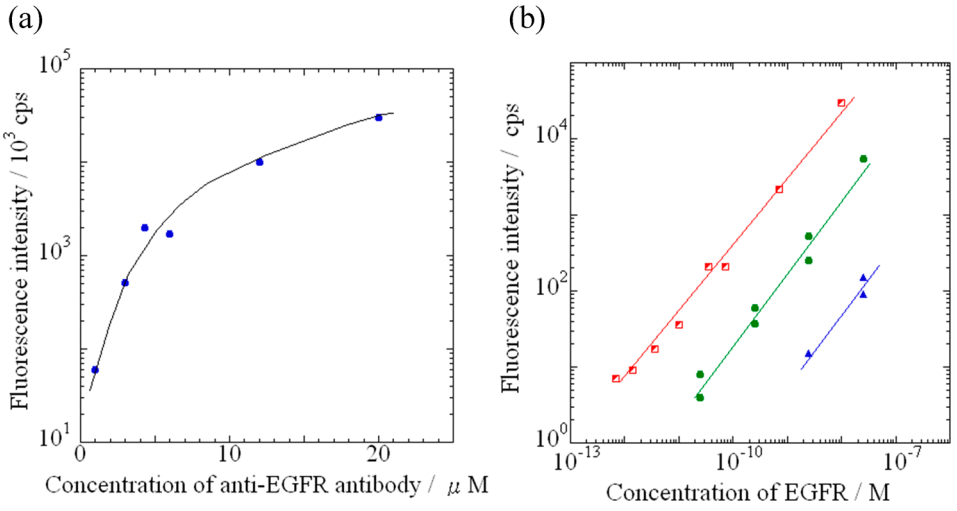

Figure 4. (a) Fluorescence intensity of 10nM cy5-sEGFR plotted for the concentration of a bispecific antibody solution injected into the chip. (b) Fluorescence intensity measured against the EGFR concentration for the plasmonic chip modified with a bispecific antibody prepared at 1 (blue triangles), 4.3 (green circles), and 20 μM (red squares).

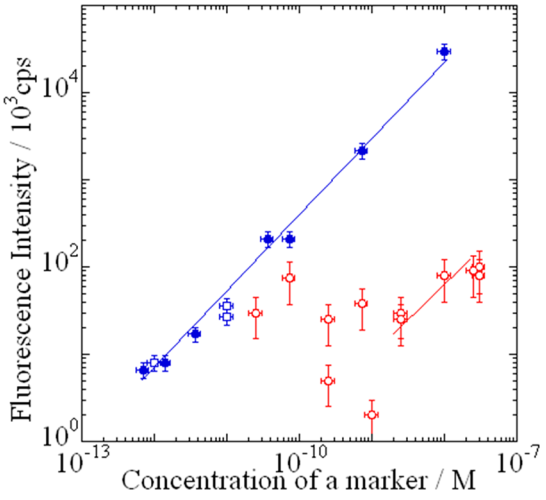

Figure 5. Fluorescence intensity plotted for sEGFR concentrations. Blue filled circles and red open circles correspond to the data on the plasmonic chip and the ZnO-coated glass, respectively. Blue open squares show the fluorescence intensity of cy5-sEGFR including 1 μM BSA. All of the data are within 20% and 50% error for the plasmonic chip and glass slides, respectively.

cy5-sEGFR solutions. The LOD was found to be 700 fM. In our plasmonic chip, the laser light irradiated from the rear panel of the chip and fluorescence was detected from the top panel of that with a PMT, and therefore the noise intensities of transmission and scattering light were reduced by the metal coating compared with that on the ZnO-coated glass slide. Furthermore, the fluorescence peak based on the reverse coupling was observed at a specific angle and was clearly classified with a scattering error. Conversely, on the ZnO-coated glass slide, it was difficult to evaluate the fluorescence intensity at sEGFR solution concentrations of <1 nM (Figure 5). sEGFR solutions with a concentration of <1 nM exhibited a nondirectional fluorescence intensity of 3×10^4 cps with a scatter of $\pm 3 \times 10^4$ cps on the ZnO-coated glass slides. The LOD in the system used here was improved from 1 nM to 700 fM with the use of a plasmonic chip instead of a ZnO-coated glass slide. On the other hand, sEGFR was quantitatively detected in the concentration range over 6 orders, from 10^{-13} to 10^{-8} M, for a plasmonic chip. For the ZnO-coated glass, sEGFR was quantitatively detected in the range of 2 orders. Consequently, the enhanced fluorescence measured on the plasmonic chip yielded a wide detection range or a high sensitivity. To date, a great deal of research related to highly sensitive tumor-marker detection has been reported.[26−31] Improvement of the detection tools such as SPR, QCM, and dissipation (QCM-D) and the enzyme-linked immunosorbent assay (ELISA) with, e.g., gold nanoparticles,[24,25] has been promoted. Although the values of the LOD reported depended on the antigen (marker) and the detection tool, they were below 1 pM (e.g., 30−50 fM) for the tumor necrosis factor α and carcinoembryonic antigen, while they were in many studies above 1 pM levels for the other tumor markers including EGFR. Therefore, the present study showing that the plasmonic chip immobilized with a recombinant bispecific antibody[8−10] detecting sEGFR up to 700 fM within 10 min under a simple operation is considered to provide significant progress. Furthermore, Arteaga reported that the serum EGFR level was below 100 pM in healthy controls and above 100 pM in women with breast cancer, respectively.[32] In this concentration range, our plasmonic chip will be efficiently available to be used as a EGFR sensor chip.

Suppression of Nonspecific Adsorption. Suppression of nonspecific adsorption, as well as highly sensitive detection, is an important factor in biosensing. The ZnO surface modified with a bispecific antibody in the phosphate-buffered saline solutions was basically expected to suppress nonspecific adsorption.[7,14] The ratios of the fluorescence intensity of cy5-labeled BSA adsorbed nonspecifically to that of cy5-sEGFR bound specifically were 0.5% and 65% for the plasmonic chip and ZnO-coated glass slide immobilized with 3 μM bispecific antibody, respectively. The large nonspecific ratio obtained in the ZnO-coated glass slide is due to the facts that the fluorescence intensity was less than 1×10^5 cps and the error obtained was very large. Furthermore, the fluorescence intensity of cy5-sEGFR specifically bound to the plasmonic chips was independent of coexistence with concentrated BSA. When 1 μM BSA (=0.066 mg mL^{-1}) was added to 10 and 1 pM EGFR solutions, the fluorescence intensities were almost in line with

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the curve drawn in Figure 5. Consequently, our detection system using a ZnO-coated plasmonic chip can be available to test human serum or whole blood including various kinds of proteins. In the future, we can develop the plasmonic chip immunosensing system to the sandwich assay; i.e., the labeled-detection antibody can be used without fluorescently labeling the antigen.

CONCLUSION

Application of the GC-SPF method with a plasmonic chip to an immunosensor contributed to the sensitive detection of markers at lower concentration. A plasmonic chip having a precise regularity and an appropriate duty ratio in a periodic structure was designed, and the sensor surface densely modified with an anti-ZnO × sEGFR bispecific antibody increased the fluorescence enhancement factor from 100 to 300 times and then improved the LOD up to hundreds of femtomolar levels. Furthermore, the interface of the ZnO-coated plasmonic chip well suppressed nonspecific adsorption. The precision of the fluorescence intensity for sEGFR detection despite the coexistence of other proteins was also observed on the plasmonic chip. Such a superior plasmonic chip was simply fabricated by UV−NIL and simple coating with silver and ZnO films, provided the enhanced fluorescence of the tumor-marker proteins: (1) under irradiation from the rear panel at a resonance angle, i.e., excitation by an enhanced electric field; (2) including a directional fluorescence, i.e., fluorescence enhanced by the reverse coupling between fluorescence and grating-coupled surface plasmon polaritons. That is, the enhanced fluorescence intensities were based on the excitation rate due to the enhanced plasmonic field and the quantum yield of the fluorophers due to the reverse-coupling mode. Our plasmonic chip interface has great potential as a clinical diagnostic chip available to the blood test because of the highly detection sensitivity, good reproducibility under the coexistence of various proteins, and rapid and simple operation.

ASSOCIATED CONTENT

Supporting Information

Measurement by SPR−SPF and chip interface immobilized with a bispecific antibody and phosphate. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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