Single-Protein Molecular Interactions on Polymer-Modified Glass Substrates for Nanoarray Chip Application Using Dual-Color TIRFM

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The immobilization of proteins and their molecular interactions on various polymer-modified glass substrates [*i.e.* 3-aminopropyltriethoxysilane (APTS), 3-glycidoxypropyltrimethoxysilane (GPTS), poly (ethylene glycol) diacrylate (PEG-DA), chitosan (CHI), glutaraldehyde (GA), 3-(trichlorosilyl)propyl methacrylate (TPM), 3'-mercaptopropyltrimethoxysilane (MPTMS), glycidyl methacrylate (GMA) and poly-*l*-lysine (PL).] for potential applications in a nanoarray protein chip at the single-molecule level was evaluated using prism-type dual-color total internal reflection fluorescence microscopy (dual-color TIRFM). A dual-color TIRF microscope, which contained two individual laser beams and a single high-sensitivity camera, was used for the rapid and simultaneous dual-color detection of the interactions and colocalization of different proteins labeled with different fluorescent dyes such as Alexa Fluor® 488, Qdot® 525 and Alexa Fluor® 633. Most of the polymer-modified glass substrates showed good stability and a relative high signal-to-noise (S/N) ratio over a 40-day period after making the substrates. The GPTS/CHI/GA-modified glass substrate showed a 13.5-56.3% higher relative S/N ratio than the other substrates. 1% Top-Block in 10 mM phosphate buffered saline (pH 7.4) showed a 99.2% increase in the blocking effect of non-specific adsorption. These results show that dual-color TIRFM is a powerful methodology for detecting proteins at the single-molecule level with potential applications in nanoarray chips or nano-biosensors.

Key Words : Nanoarray protein chip, Dual-color TIRFM, Single-molecule detection, Polymer-modified glass

Introduction

Since the development of microarray chip technology, nanoarray technology has become an invaluable tool for the rapid screening of DNA and protein samples.[1-7] Nanoarray chip technology allows a large number of target molecules to be detected in an ultra-small area through fast, accurate and high-throughput screening. However, despite the success of nanoarrays for DNA chip applications, a similar trend for protein chips has not occurred due to difficulties in the prevention of non-specific binding and the detection of individual protein molecules on a single-molecule level. A protein probe adsorbed at the wrong position on the chip substrate causes signal interference, which can lead to incorrect detection. The proper surface modification of a nanoarray protein chip can minimize the uncontrolled non-specific adsorption of protein molecules. Generally, the interaction between two protein molecules is indicated by the colocalization of the two molecules using bulk and fluorescence microscopy. However, conventional fluorescence can only visualize the assembly of two protein molecular species when many protein molecules of each species are used. Moreover, the ability to detect various protein molecular interactions will be also virtually impossible if the fluorescence lifetime of the molecule employed is short. Hence, there is a need for new detection technology or methods to observe individual single-protein molecules on various substrates in order to determine the optimum substrate condition for efficient single-protein molecule immobilization and the precise binding condition of individual protein molecules on a nanoarray chip surface.

Single-molecule detection (SMD) is an emerging technique for examining protein interactions *in vivo* and *in vitro*.[8] Among the SMD techniques, total internal reflection fluorescence microscopy (TIRFM) has been used successfully at solid/liquid interfaces due to the small fluctuations and low background noise.[9,10] Conventional TIRFM techniques are mainly used a single specific light source with a single-wavelength laser as the excitation energy source. However, TIRFM with a single-wavelength can create some confusion while attempting to observe the complimentary binding between the target protein molecules and capture protein molecules in a mixture because the single-wavelength excitation causes only single-color fluorescence imaging. Recently, Kusumi *et al.* reported a method for the synchronous detection of two full Ecad-GFP and Aleax633-αEcad-Fab images in living cells in two different colors using an objective-type dual-color TIRFM with two high-sensitivity cameras.[11] The routing of organelles within cells was also examined using dual-color TIRFM with different two lasers, two cameras and mechanisms.[12] However, although Simon *et al.*[13,14] and Rutter *et al.*[15] examined the role and mechanisms of the proteins in cells using dual-color TIRFM with different two lasers and one camera, they acquired the

images at 2-5 Hz with a long exposure time > 150 ms. Moreover, they did not show the potential use of the dual-color TIRFM technique in nanoarray protein chip applications .

In this study, a prism-type dual-color TIRFM system with two individual lasers and just one high-sensitivity camera was developed in order to examine the protein molecular interactions on representative polymer-modified glass substrates, which are mainly used at microarray chip fields. There was no time-delay and mechanical deviation because the images of the individual single-protein molecules were obtained using a single CCD camera only and without the need to move the sample. The optimum conditions for applications to a nanoarray protein chip were quantified in an attempt to reduce the non-specific binding and increase the high efficient immobilization. The effective blocking conditions for decreasing the non-specific adsorption of protein molecules on the polymer-modified glass substrates was also examined at the single-molecule level with an exposure time of 10 ms and an acquisition time of 10 Hz.

Experimental Section

Chemicals and Reagents. The 3-Aminopropyltriethoxysilane (APTS), 3-glycidoxypropyltrimethoxysilane (GPTs), glycidyl methacrylate (GMA), poly (ethylene glycol) diacrylate (PEG-DA, M_N = 258), chitosan (CHI, 75-85% deacetylated chitin, M_N = 50 000-190 000), 3'-mercaptopropyltrimethoxysilane (MPTMS), acetic acid, poly-*l*-lysine (PL), streptavidin, phosphate buffered saline (PBS, pH 7.4) and phosphate buffered saline with Tween 20 (PBS-T, pH 7.4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The glutaraldehyde (GA), 3-(trichlorosilyl)-propyl methacrylate (TPM) and Maleimie PEO_2-biotin were obtained from Fluka Chemical Co. (Milwaukee, WI, Germany) and Pierce (Rockford, USA), respectively. The chloroform, methanol and ethanol were supplied by Fisher Scientific (Fair Lawn, NJ, USA). The parapin oil (density = 0.830 g mL^{-1}) was acquired from DC Chemical Co. (Seoul, Korea). The blocking solution was used with a Top Block (Fluka Chemical Co., Milwaukee, Germany), bovine serum albumin (BSA, Sigma) and PEG maleimide (ID Biochem Inc., Seoul, Korea). The cover glass was purchased from Dow Corning (Midland, Michigan, USA). The 10 mM PBS (0.138 M NaCl and 0.0027 M KCl in 1 L water at 25 °C) and 10 mM PBS-T (0.138 M NaCl, 0.0027 M KCl and 0.05% Tween 20 in 1 L water at 25 °C) were dissolved in ultra-pure water (> 15 MΩ). All the buffer solutions were filtered through a 0.2-μm membrane filter (MilliQTM/Milli-RO Water System) and photo-bleached overnight using a UV-B lamp (G15T8E, 280-315 nm, Philips, The Netherlands) prior to use.

Protein Sample Preparation. Actin from rabbit muscle, actin from rabbit muscle-Alexa Fluor® 488 conjugate and Alexa Fluor® 633-conjugated goat anti rabbit IgG were purchased from Molecular Probes (Eugene, OR, USA). The anti-actin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The actin from the rabbit muscle-Alexa Fluor 488® conjugate and anti-actin were incubated for 60 min at 4 °C to produce a secondary antibody reaction between the immunoassay methods. The Alexa Fluor® 488 conjugated actin-anti actin solution, in which the rabbit muscle-Alexa Fluor® 488 conjugate was joined to anti-actin, was added to the Alexa Fluor® 633 conjugated goat anti rabbit IgG. The actin biotin labeled-protein sample was purchased from Aprogen Inc. (Daejeon, Korea). The fluorescent dye labeling for use on the MPTMS coated substrates was performed using Qdot® 525. The antibody conjugation kit reagents (Quantum Dot Corp., Hayward, CA, USA) were used according to the procedure reported in the manual.[16] All protein samples were prepared at a concentration of 1 μM in a 10 mM PBS buffer. For the SMD experiments, the protein samples were further diluted to 1 pM-10 fM with 10 mM PBS immediately before beginning the experiments.

Polymer Modifications of Glass Substrate. A variety of coated substrates were prepared according the methods reported elsewhere.[17-21] Figure 1 shows a schematic diagram of the method used to prepare the representative polymer-modified glass substrates. A bare cover glass was cleaned by

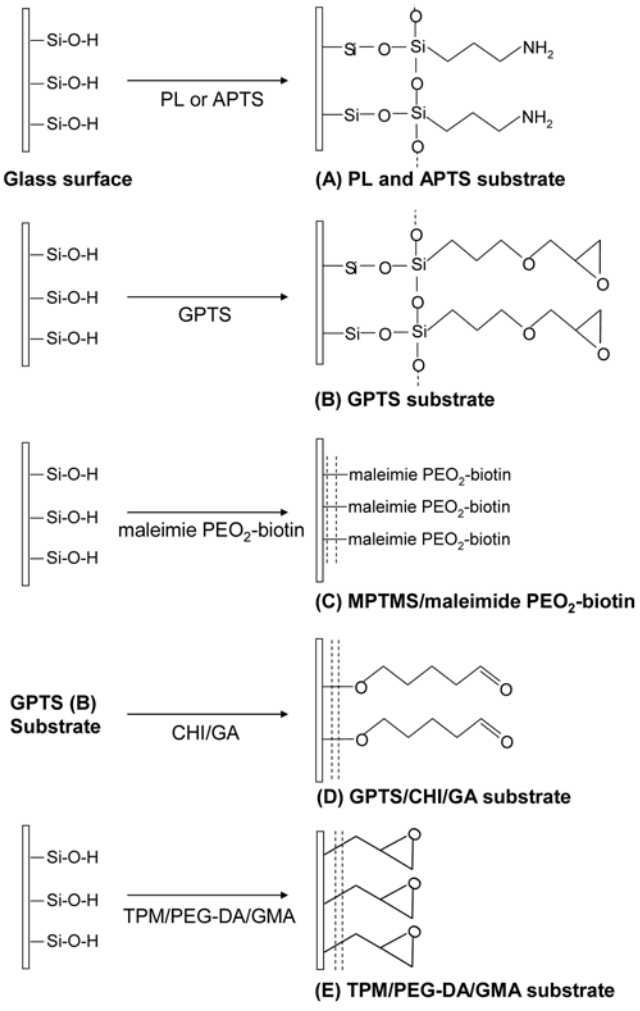

Figure 1. Schematic diagram of the preparation of the representative polymer-modified glass substrates.

sonification in a mixture of methanol and deionized water (1:1, v/v) at room temperature for 3 h. The cover glasses were then rinsed thoroughly with deionized water, and then blow-dried with N_2 gas. The PL coating was carried out in a solution containing 0.01% PL in 10 mM PBS at room temperature.[17,18] The non-adsorbed coating material was removed by rinsing the PL-modified glass substrate with PBS and deionized water. The PL-modified substrate was then baked in an oven at 50 °C for 30 min. The APTS coating was carried out in a solution of 3-5% APTS in ethanol for 1 h.[17,19] The non-adsorbed APTS was removed by sonicating the APTS coating surfaces in ethanol for 10 min. The APTS-modified cover glasses were rinsed thoroughly with ethanol and blow dried with dry N_2 gas. Finally, the APTS-modified substrate was baked in an oven at 110 °C for 15 min. The GPTS coating solution was prepared with 5% GPTS in chloroform and 2.5% GPTS in a solution containing 5% acetic acid in ethanol. The 5% GPTS coating was carried at 50 °C for 12 h, and the 2.5% GPTS coating was carried at room temperature for 1 h. The 5% GPTS-modified cover glasses were sonicated in chloroform for 10 min, and the 2.5% GPTS-modified glasses were sonicated in ethanol for 10 min to remove the excess coating material. The GPTS-modified substrate was rinsed thoroughly with ethanol and blow dried with N_2 gas. The TPM coating was covalently attached to the cover glasses in order to increase the level of adhesion between PEG-DA/GMA and glass.[20] The TPM coating was performed in a solution of 1% TPM in paraffin oil (DC Chemical Co., Seoul, Korea) at room temperature for 15 min. The coated substrate was rinsed with ethanol, and baked in oven at 95 °C for 30 min. The MPTMS coating was carried out using a two-step silanization procedure.[21] Briefly, the cover glasses were silanized *via* evaporation from a 2 μL drop of neat liquid MPTMS at 120 °C for 10 min in a covered 150 mL glass jar, followed by extensive washing with deionized water. After drying with nitrogen gas, the glass was further silanized for an additional 10 min with a fresh 2 μL drop of MPTMS under the same conditions. The MPTMS-modified substrate was baked at 100 °C for 16 h. The MPTMS-modified substrate was coupled with maleimide PEO_2-biotin (25 mg/mL) at room temperature to allow streptavidin-biotin binding, rinsed with deionized water, and blow-dried with N_2 gas. The GA and CHI coatings were coupled to the epoxy groups on the GPTS-modified substrates.[19] The GA coating was carried out using a 5% GA solution in 10 mM PBS at room temperature for 1 hour, rinsed in deionized water, and blow dried with N_2 gas. The CHI coating was carried out for 12 h at room temperature in a 1% CHI solution in deionized water, to which acetic acid was added until the solution became clear. After the coating, the CHI-modified substrate was sonicated in deionized water for 20 min, rinsed thoroughly in deionized water, and blow dried with N_2 gas. The PEG-DA/GMA coating solution was made by mixing PEG-DA and a photoinitiator (GMA) at volume ratio of 8:2. The mixtures were vortexed for 1 min and kept in the dark prior to use. The substrates were irradiation with a UV lamp for 30 min. The irradiated substrates were rinsed with water and blow dried with N_2 gas. All the polymer-modified cover glasses were stored in vacuum bottles.

Single-Protein Molecules Immobilization. An inkjet array method using a micro pipette was used to chemically attach the protein molecules to the polymer-modified glass surfaces.[22] 250-300 nL Alexa fluor® 633 goat anti-rabbit IgG was introduced to most polymer-modified glass substrates, which were then incubated at 3.5-4.0 °C for 30 min. However, on the MPTMS-modified substrates, the streptavidin was first incubated at 4.0 °C for 30 min, rinsed with 10 mM PBS-T, and dried in a nitrogen atmosphere. The streptavidin-bound cover-slip surface was then incubated with Qdot® 525 conjugated actin-biotin under the same conditions because of the binding of the biotinylated protein (Qdot® 525 conjugated actin-biotin).

Blocking of Non-Specific Adsorptions. Top Block, BSA and PEG-maleimide were used to improve the blocking efficiency and prevent non-specific binding. In the MPTMS-treated glass substrate, PEG-maleimide was used as a blocking solution for the non-specific adsorption of protein molecules. Top Block and BSA were used as a blocking solution in the other polymer-modified glass substrates. All the blocking solutions of 1% Top Block, 1% BSA and 1 mg mL^{-1} PEG-maleimide were made using 10 mM PBS. Blocking solutions were applied to capture the proteins immobilized on the chip surface. The chip was then incubated for 30

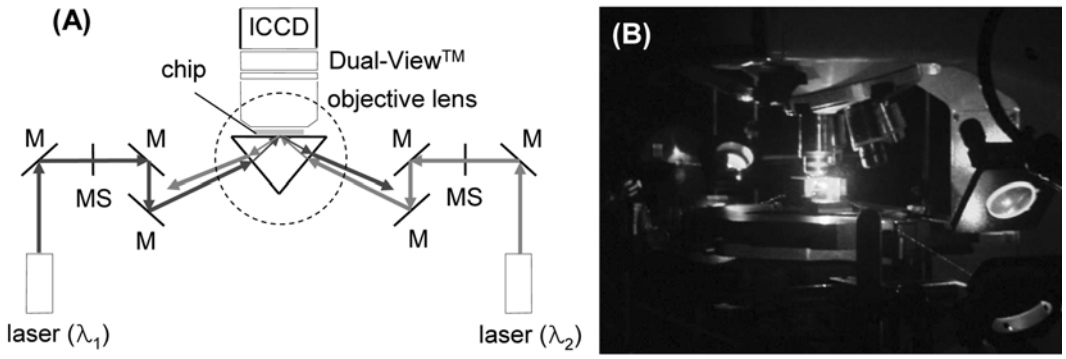

Figure 2. (A) Schematic diagram and (B) image of the experiment setup of the dual-color TIRFM system used for the immobilization and interactions of the individual single-protein molecules on various polymer-modified glass protein chips. Indicate: M, mirror; MS, shutter; λ, Laser source.

min at 3.5-4.0 °C in a humid chamber, washed with a 10 mM PBS-T solution, and dried in a nitrogen atmosphere.

Dual-Color TIRFM System. The basic experimental dual-color TIRFM setup was similar to those described in previous reports of single-wavelength TIRFM systems except for two individual lasers and one Dual-View™.[23,24] As shown in Figure 2, two different individual lasers, one is a wavelength tunable argon ion laser (Max output power 150 mW at 488 nm; Melles Griot, Irvin, CA, USA) and the other one is 635 nm fiber coupled laser (maximum output power 100 mW at 635 nm; B&W TEKINC, Newark, DE, USA), were used as the excitation light sources. An upright Zeiss Axioskop2 microscope (Zeiss, Germany) equipped with a Dual-View™ (Optical Insight, LLC, Tucson) was used for most investigations. The Dual-View™ was mounted between the objective lens and the CCD camera. The Dual-View™ filter cube is consisted of a fixed mirror, a dichroic filter (565dcxr, Chroma technology Corp., Rockingham, USA) and two emission filters (D680/35 and D535/40, Chroma Technology Corp., Rockingham).[25] A Zeiss 100 ×/1.3 N.A. Plan-Neofluar® microscope objective lens (Zeiss, Germany) was used. A Pentamax 512-EFT/1EA intensified CCD camera (ICCD, Princeton Instruments, Princeton, NJ, USA) was mounted on top of the microscope. The laser beams were transmitted through an optical pinhole to eliminate the extraneous light and plasma line as well as to reduce the laser diameter. An Uniblitz mechanical shutter (model LS2Z2, Vincent Associates, Rochester, NY, USA) was used to block the laser beam when the camera was off in order to reduce photo-bleaching. The shutter was controlled by a model VMM-D1 shutter driver (Vincent Associates). The experimental timing was controlled using a Stanford Research System model DG-535 four channels digital delay/pulse generator (Stanford Research Systems, Inc., Sunnyvale, CA, USA). The ICCD camera was triggered at time 0 ms with a 10 ms duration TTL pulse. The sampling frequency was 10 Hz with a shutter driver set to 10 ms exposure and 90 ms delay. The fluorescence from the individual single-protein molecules was passed through a Dual-View™ filter box in order to eliminate the unnecessary light and obtain different images of the individual different single-protein molecules labeled with the different fluorescent dyes, Alexa Fluor® 488, Qdot® 525 and Alexa Fluor® 633 on the one CCD camera. The evanescent wave excitation geometry was also similar to that previously reported.[23,24] Briefly, a polymer-modified substrate was placed on the hypotenuse face of a right-angle fused silica prism (Melles Griot, Irvine, CA, USA; A = B = C = 2.54 cm, refraction index, n = 1.463). The protein sample was placed on the polymer-modified glass chip and a bare cover glass was placed on the sample. The area between the polymer-modified substrate/prism and the bare cover glass/objective lens were index-matched with a drop of immersion oil (Zeiss, Germany). The two different individual laser beams were directed through the prism toward the polymer-modified glass chip in the opposite direction. All incidence angles, θ_i, were slightly greater than 69°. The image collections of the individual single-protein molecules and data processing were carried out using WinView/32™ (Version 2.5.14.1, Downing town, PA, USA) and MetaMorph 6.3 (Universal Imaging Co., Downingtown, PA, USA) software.

Observation of Single-Protein Molecules on the Substrates. Figure 3 shows the overall experimental procedure for observing the protein-protein interactions on the polymer-modified glass substrate. The polymer-modified glass substrates were evaluated after washing the chip with 10 mM PBS-T followed by drying in a nitrogen atmosphere. The sample on the chip was monitored after introducing 4 μL of a 10 mM PBS solution. Direct observations of the individual protein molecules and immunoassay on the chip were usually carried out as follows: 2 μL of the target proteins (Alexa Fluor® 633 goat anti rabbit IgG) were introduced onto the chip surface and incubated for 30 min at 3.5-4.0 °C. The non-bound target protein molecules on the chip surface were removed by washing with 10 mM PBS-T. Immediately before imaging, the sample was rehydrated with 2 μL of 10 mM PBS and covered with another cover glass. The individual protein molecules on the chip were then observed using the dual-color TIRFM system.

Results and Discussion

Dual-Color TIRFM System for Individual Protein Molecule Detection. Various single-molecule detection methods, including the single-molecule fluorescence resonance energy transfer (FRET) technique developed by

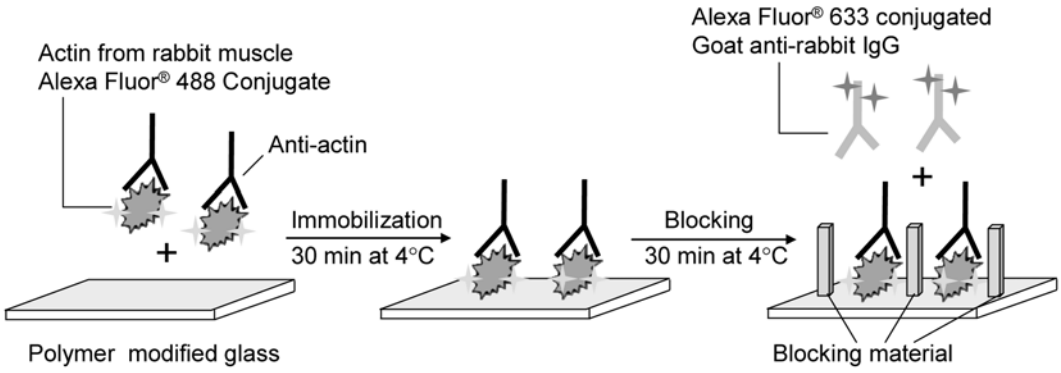

Figure 3. The overall experimental procedure for observing the protein-protein interactions on the polymer-modified glass substrate.

Murakoshi *et al.*,[26] are the only ways of directly visualizing the single transfer process, the colocalization of individual molecules and the possible involvement of scaffolding proteins in these processes.[11] However, in this study, an upright dual-color TIRFM system was developed with two individual lasers and only one intensified CCD camera using prism-type total internal reflection. The fluorescence emission signal from the protein sample was separated chromatically using a Dual-View™, which consisted of a dichroic filter and two emission filters. After obtaining the two split images separately at different regions of a single CCD camera with a frame rate of 10 Hz, the images were overlaid and analyzed to determine the surface characterization and efficient immobilization of the individual protein molecules, as well as to track the colocalization and molecule-molecule interactions on the chip at the single-molecule level. The captured protein molecules were observed only at their original emission wavelength of 535 nm when only Alexa Fluor® 488 conjugated actin-anti actin (or Qdot® 525 conjugated actin-biotin), as a capture protein molecule, had been loaded onto the polymer-modified glass chip substrates. No images (or intensity) of the protein molecules were obtained at the emission wavelength of the target protein molecules labeled with Alexa Fluor® 633 conjugated goat anti rabbit IgG at its original emission wavelength of 680 nm when the Alexa Fluor® 488 conjugated actin-anti actin (λ_{ex} = 488 nm) was mixed with the target protein molecule labeled with Alexa Fluor® 633 conjugated goat anti rabbit IgG (λ_{ex} = 635 nm). This suggests that there was no contribution from FRET between the Alexa Fluor® 488 conjugated actin-anti actin and Alexa Fluor® 633 conjugated goat anti rabbit IgG on the polymer-modified glass chip.

Characterization of the Polymer-Modified Glass Substrates. Various polymer-modified glass substrates were identified using static contact angle measurements (Seo Phoenix 300), atomic force microscopy (AFM; MultiMode and BioScope, Digital Instruments, USA) and X-ray/ultraviolet photoelectron spectroscopy (XPS; AXIS-NOVA, Kratos Inc, USA). Although there was little change, the results were almost the same in the acceptable error ranges compared with those reported earlier (data not shown). Therefore, the protein molecule immobilization and molecular interactions on polymer-modified glass substrates such as APTS, GPTS, PEG-DA, CHI, GA, TPM, MPTMS and PL were evaluated at the single-molecule level using the dual-color TIRFM system. The protein molecules were immobilized on the APTS-, GPTS-, CHI- and PL-modified glass substrates through physical adsorption. Although the interactions caused significant non-specific binding, the protein molecules were simply adsorbed on the amine coated-substrates by a hydrophobic force and electrostatic attraction.[17,18,27-30]

The number of immobilized Alexa Fluor® 633-goat anti-rabbit IgG protein molecules, as an example capture protein molecule, on the amine-modified substrate increased with increasing the stock time after making the chips. The relative S/N ratio on the amine-modified glass chip was 1.5-2.5 times lower than the other polymer-modified substrates

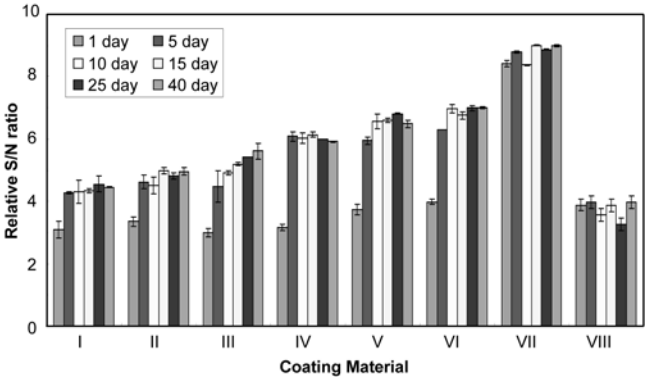

Figure 4. Relative signal-to-noise (S/N) ratio of the various polymer-modified glass substrates at the single-molecule level using dual-color TIRFM. Sample, 1 pM Alexa Fluor® 633-goat anti-rabbit IgG on most polymer modified-substrates. 1 pM Qdot® 525 conjugated actin-biotin only at the MPTMS-modified glass (XI); buffer, 10 mM PBS and 10 mM PBS-T; incubation for 30 min at 4.5 °C; ICCD exposure time, 10 ms. . Indicate: I, PL; II, 3% APTES; III, GPTS; IV, GPTS/GA; V, GPTS/CHI; VI, GPTS/CHI/GA; VII, MPTMS and VIII, TPM/PEG-DA/GMA. *The error bars indicate the standard deviations of eleven measurements.

(Figure 4). However, the relative S/N ratio on the secondly CHI-modified surface using the GPTS-modified substrate (GPTS/CHI, Figure 4V) was approximately 45% higher than those on the only amine-modified substrates such as the APTES and PL coating (Figures 4I and II). An amine-coated substrate generally provides sufficient immine bridges with proteins, even though it causes steric hindrance between the protein molecules and surface due to the shortness of the arms of the amine coating materials.[19,31] The amine-modified substrate with many amine groups enhances the electrostatic interaction with *the* approaching proteins. The high intensity of the S/N ratio of Alexa Fluor® 633-goat anti-rabbit IgG on the GPTS/CHI-modified chip substrate originated from the strong interaction between the protein molecules and substrate because the level of steric hindrance was decreased by the long arm length and the many amine groups on the modified-substrate surface.

The substrates in previous studies had been characterized at different concentrations of the target protein samples, and the relative signal intensity was measured without considering the non-specific adsorption.[17,32,33] The significant non-specific adsorption and binding was measured a concentration above 500 fM.[34] However, a higher number of protein molecules were observed on the amine-modified substrate than on the other polymer-modified substrates because the protein on the amine-modified substrates had not been immobilized by covalent binding but by simple physical adsorption. In addition, the number of immobilized protein molecules (Alexa Fluor® 633-goat anti-rabbit IgG) on the 3% and 5% APTES-modified chip substrate was relatively unchanged, and the S/N ratio was 3.6% higher on the 5% APTES substrate (data not shown). This suggests that the concentration of the coating material did not have the largest effect on the immobilization of the single-protein molecules on the polymer-modified glass substrates at the single-

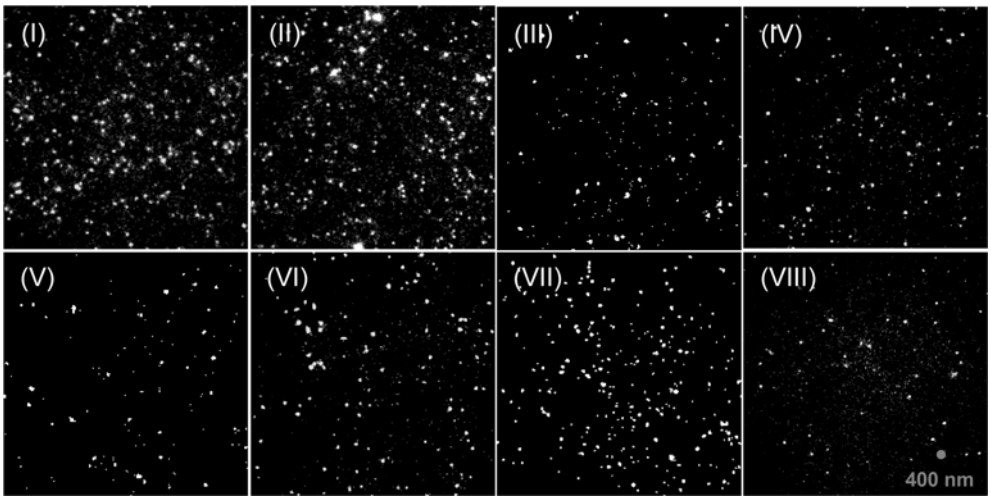

Figure 5. Representative images of the single-protein molecules on the various polymer-modified substrates. The other dual-color TIRFM conditions were the same as those reported in Figure 4.

molecule level. The protein molecules were immobilized on an aldehyde/epoxy-modified substrate by covalent bonding between the primary amine of the protein and the substrate to develop a Schiff's base.[17]

The GPTS-modified substrates showed better stability than those of PL- and APTES-modified substrates within 10-40 days after making the glass chip (III-VI in Figure 4). The results at the single-molecule level are in accordance with the previous results showing that the relative signal intensity of the GPTS-treated substrate increased continuously for 1 month at 4 ^{o}C at the bulk level.[35] Although the efficiency of immobilization of the individual Alexa Fluor® 633-goat anti-rabbit IgG protein molecules on the aldehyde-modified substrate was approximately 25% lower than that on the amine-modified substrate, the S/N ratio increased by 25-100%. There was no aggregation on the GPTS-treated substrate (III-VI in Figures 5). It was concluded that this phenomenon was the result of covalent bonding between the individual Alexa Fluor® 633-goat anti-rabbit IgG protein molecules and the aldehyde groups of the substrate, which is a site-directed immobilization method.[36-38] The key points in making a good array chip are to increase the binding capacity of the individual molecules on the substrate and have a high-throughput manufacturing and screening procedure.[32] Since the protein molecule does not have a negatively charged-phosphate backbone, unlike a DNA molecule, coating materials with an upper-directed active site need to be used to make a high efficient array chip.

Kusnezow *et al.* reported that the signal intensity of an immobilized protein increased with increasing length of the cross-linker on APTES- and MPTMS-modified substrates.[27] In single-molecule detection experiments using dual-color TIRFM, the number of immobilized protein molecules increased with increasing cross-linker length on the GPTS-modified substrate. As shown in Figures 4VII and 5VII, the MPTMS-modified substrates contained the highest number of immobilized protein molecules (Qdot® 525 conjugated actin-biotin) and S/N ratio compared with the other polymer-modified substrates at the single-molecule detection. The MPTMS-modified substrate is mainly used in dip pen nanolithography in atomic force microscopy (AFM), even though the substrate requires a complicated series of steps to modify the protein using biotin and streptavidin.[21] However, there was a problem in demonstrating a direct interaction between the individual native protein molecules and the polymer on a glass substrate. This is because the MPTMS polymer does not interact with the individual native protein molecules.

The PEG hydrogel-modified glass substrate made by photo-polymerization was also evaluated on the single-protein molecule level using dual-color TIRFM. In general, the substrate modified by photo-polymerization shows a high RMS roughness compared with other polymer-modified substrates.[19,31] As shown in Figures 4VIII and 5VIII, the immobilized protein, the number of molecules and the relative S/N ratio were also lowest values at the single-molecule level using the new detection system.

Blocking of Non-Specific Adsorptions. The non-specific adsorption of individual protein molecules on the polymer-modified glass substrates depends on the blocking (or passivation) conditions (Figure 6). Recently, a bio-material method was mainly used to block the non-specific adsorption,[17,39] of which the size affects the S/N ratio due to steric hindrance. Therefore, 5% APTES-, GPTS/CHI/GA- and MPTMS-modified glass chips were selected because of the superior S/N ratio and sensitivity. The blocking effect of 1% Top Block (MW = 3 kDa), 1% BSA (MW = 60 kDa) and 1 mg/mL PEG-maleimide were measured as a function of their sizes. The reagents showed blocking efficiencies of 97.9-99.2%, which is similar to each modified glass substrate (B2 in Figure 6). The shorter blocking reagent had a higher blocking effect of the non-specific adsorption as follows: Top Block > BSA > PEG-maleimide. Compared with the BSA and PEG-maleimide, Top Block had a 64.1% and 31.7% higher blocking effect, respectively (Figure 6A). This shows that the smaller blocking reagent has stronger blocking effect on non-specific adsorption at the single-

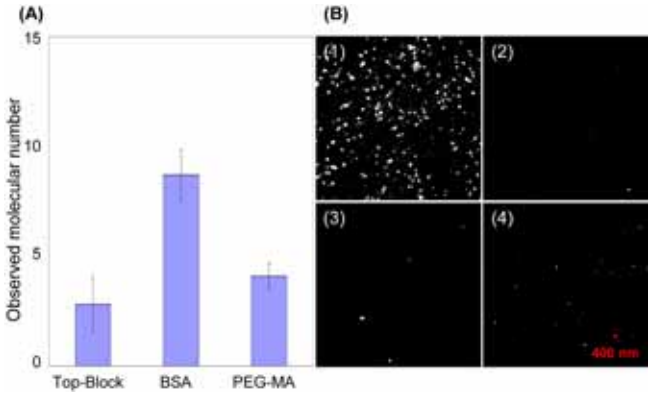

Figure 6. Histograms showing the average number of observed protein molecules on the 5% APTES-, GPTS/CHI/GA- and MPTMS-modified glass substrates (A) and representative TIRFM images of the single-protein molecules (B) on only the GPTS/CHI/GA-modified glass substrate depend on various block conditions of non blocking (B1), 1% top block (B2), 1% BSA (B3) and 1 mg mL^{-1} PEG-maleimide (B4). Sample, 1 pM Alexa Fluor® 633-goat anti-rabbit IgG at the GPTS/CHI/GA-modified glass substrates. 1 pM Qdot® 525 conjugated actin-biotin at the MPTMS-modified glass substrates; buffer, 10 mM PBS and 10 mM PBS-T; blocking incubation for 20 min at 4.5 °C. The other dual-color TIRFM conditions were the same as those reported in Figure 4. *The error bars indicate the standard deviations of the eleven measurements.

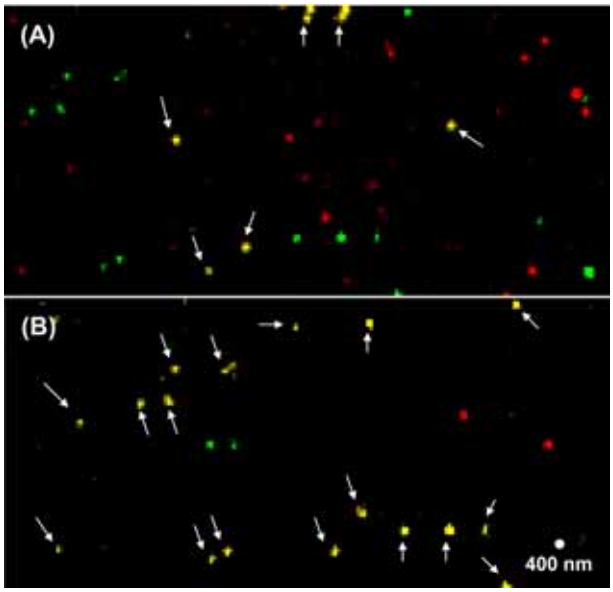

Figure 7. Dual-color TIRFM analyses of the protein-protein interaction (A) without and (B) with the blocking reagent of 1% Top-Block in PBS on the GPTS/CHI/GA-modified glass protein chip. Protein samples, 1 pM Alexa Fluor® 633-goat anti-rabbit IgG (target protein and Alexa Fluor® 488 conjugated actin-anti actin (capture protein); buffer, 10 mM PBS; washing buffer, 10 mM PBS-T. Indicate: Red dot, Alexa Fluor® 633 conjugated goat anti rabbit IgG; green dot, Alexa Fluor® 488 conjugated actin-anti actin; yellow dot (arrows), binding of the two different protein molecules on the chip substrate. The other dual-color TIRFM conditions were the same as those described in Figure 4.

molecular level.

Figure 7 shows the representative dual-color TIRFM analyses of Alexa Fluor® 633 conjugated goat anti rabbit IgG and Alexa Fluor® 488 conjugated actin-anti actin as a model protein-protein interaction at the optimum interaction condition on the GPTS/CHI/GA-modified glass chip substrate with and without blocking reagent of 1% Top-Block. Clear fluorescence contrast was obtained from the Alexa Fluor® 633 conjugated goat anti rabbit IgG samples after an interaction with the actin from rabbit muscle Alexa Fluor® 488 conjugate. The binding of the two different protein molecules on the chip substrate had a yellow color due to the co-localization of each protein molecules with different emission colors (*i.e.* red and green). At the optimum condition with the blocking reagent (Figure 7B), the binding efficiency of the protein-protein interaction with Alexa Fluor® 633-goat anti-rabbit IgG, anti-actin and Alexa Fluor® 488 conjugated actin was calculated at a concentration of 1 pM. A concentration of 1×10^{12} M means there are 180 600 molecules per 300-nL volume, or 7.07×10^6 μm^2 area. The detection window for dual-color TIRFM is 1.46×10^4 μm^2. In this small detection area and low concentration, 319 ± 1.8 (mean ± standard deviation) individual protein molecules were observed, which indicates a precision of 85.5 ± 0.56%. This shows that almost 90% of the single-protein molecules could be detected using the dual-color TIRFM system.

Conclusions

A prism-type dual-color TIRFM system was developed to examine the single-protein molecule immobilization and interactions on various polymer-grafted glass chip substrates. The dual-color TIRFM system with two individual laser beams and one high-sensitivity camera was used for the simultaneous dual-color detection of the interactions and colocalization of the captured and target protein molecules labeled with the different fluorescent dyes *e.g.* Alexa Fluor® 488 and Alexa Fluor® 633 without any time-delay or need to move the sample. The polymer-modified glass substrates coated with APTS, GPTS, PEG-DA, CHI, GA, TPM, MPTMS and PL were used to determine their potential applications in nanoarray protein chips using the dual-color TIRFM. The highest number of immobilized protein molecules and S/N ratio were observed on the MPTMS-modified glass chip substrate at the single-molecule level. However, the MPTMS-modified glass substrate required complicated steps to modify the protein molecules with biotin and streptavidin, which did not show the direct observation of the interaction between the individual protein molecules and polymer-modified substrate. The amine-modified substrate also showed a poor efficiency for applications to a nanoarray protein chip due to the relatively low S/N ratio and poor stability. Although the number of immobilized single-protein molecules on the aldehyde-modified substrates was relatively low, these substrates can be applied to nanoarray protein chips because of the high S/N ratio and the possibility of the direct immobilization of the individual protein molecules with the aldehyde group on the substrate surface. In summary, the GPTS/CHI/GA-modified glass chip substrate had the highest efficiency because of its high S/N

ratio, stability and direct observation of the immobilization of individual single-protein molecules. These results are expected to provide an important technique for the development of a nanoarray protein chip. Although dual-color TIRFM was not applied directly to a nano-patterned protein array chip, it is expected that its applications can be extended to a wide range of biochip substrates that are nano-patterned with an assortment of individual biological molecules.

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