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Note

Adsorption of TMRbiocytin on a Bovine Serum Albumin (BSA) Coated Glass Coverslip Using Fluorescence Lifetime Imaging

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Fluorescence lifetime imaging (FLIM) is a powerful technique used in various scientific fields, particularly biology, chemistry, and materials science.[1,2] Because FLIM simultaneously measures not only the fluorescence intensity (extensive property) but also the fluorescence lifetime (intensity property), it provides more information about the surrounding environment through the fluorescent material than imaging, which relies only on fluorescence intensity.[3] FLIM is applied in various scientific fields because of its ability to provide unique insights into molecular environments, interactions, and dynamic processes. The adsorption of chemicals onto a protein surface involves the attachment or binding of molecules to the protein surface. This phenomenon occurs because of various interactions between compounds and protein surface residues. The adsorption of chemicals onto the surfaces of proteins can lead to various outcomes. This may be a desirable effect in a drug delivery system that prolongs the action of a drug by attaching it to the surface of a protein carrier.[4] Interactions between proteins and chemicals are influenced by several factors. First, electrostatic interactions occur, in which charged groups on the surface of a protein attract oppositely charged ions or molecules. For example, positively charged amino acid residues on the surfaces of proteins attract negatively charged ions or molecules. Second, the hydrophobic portion of the molecule tends to interact with the hydrophobic regions on the protein surface. These interactions are particularly important when hydrophobic residues are exposed on protein surfaces. Van der Waals forces are individually weak but contribute to adsorption in large numbers. TMR biocytin is a fluorescent substance formed by a lysine bond between tetramethylrhodamine (TMR) and biotin. TMR biocytin is used to study cell–cell, and cell–liposome fusion, membrane permeability, and cellular uptake during pinocytosis. TMR biocytin can be detected using streptavidin and is an effective neural tracer in living tissue.[5] In this experiment, we investigated the adsorption of TMRbiocytin on the BSA-coated glass surfaces using fluorescence lifetime imaging. To coat BSA on a glass coverslip, BSA was dissolved in 10 mM $NaHCO_3$ (pH 8.3, 150 μM) and this solution was incubated overnight on a glass coverslip treated with a plasma cleaner. BSA coating on glass surfaces has been studied by several researchers, and it has been revealed that if BSA at the concentration used in this experiment is exposed to the glass surface for a long time, nearly the entire surface will be covered with BSA.[6-8] *Fig*. 1 shows the fluorescence decay of TMRbiocytin adsorbed onto a glass coverslip coated with BSA. The adsorption of BSA on the glass coverslip surface was confirmed using the fluorescence lifetime of TMRbiocytin. When TMR is directly adsorbed on the glass surface, an electronic transition occurs and the fluorescence lifetime

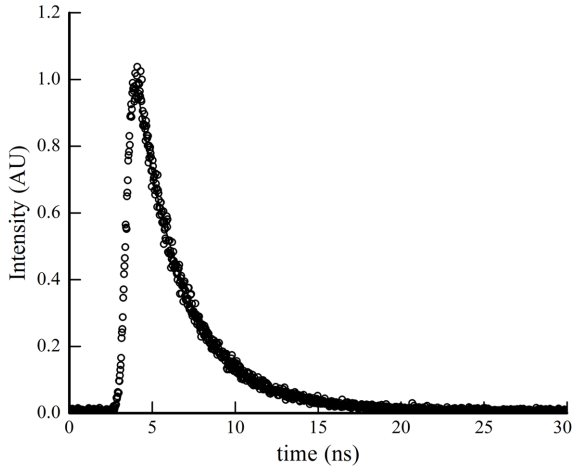

***Figure* 1.** Fluorescence decay of TMRbiocytin adsorbed on BSA coated on a glass coverslip.

is shortened.[9] According to the fitting, the fluorescence lifetime was 2.9 ns, which was the same as the fluorescence lifetime of TMR measured in solution. This indicates that TMRbiocytin was not directly adsorbed to glass but was adsorbed to the BSA surface. We cannot rule out the possibility that a very small amount of TMRbiocytin may be directly adsorbed to the glass surface on a surface not covered with BSA at the single molecule level, but the impact on the experimental results is extremely limited. The degree of adsorption of TMRbiocytin to the BSA surface was investigated together with TMR. The same concentration (1 μM) of each of TMR and TMRbiocytin were incubated on a glass coverslip coated with BSA for the same time. After a certain period, TMR and TMRbiocytin that were not adsorbed on the surface were washed off, and the surface was scanned using a confocal microscope. Because most of the fluorescence signals obtained from out-of-focus were removed by the confocal pinhole, only the fluorescence signals of TMR adsorbed on the surface could be obtained.[10,11]

Fig. 2 shows fluorescence imaging of TMR and TMRbiocytin adsorbed on the surface obtained by incubating 1 μM of TMR and TMRbiocytin on a glass coverslip coated with BSA for 20 min using the aforementioned method. It can be seen that a much larger amount of TMRbiocytin was adsorbed on the BSA surface compared to TMR. Because TMR has both a carboxyl group and an amine group, its charge varies depending on the pH. In this experiment, a $NaHCO_3$ buffer solution (pH 8.3) was used; therefore, the amine group had a positive charge, and the carboxyl group had a negative charge. The isoelectric point of BSA is pH 4.5–4.8; therefore, at pH 8.3, it has an overall negative charge.[12,13] Additionally, because it has a three-dimensional structure and is already adsorbed on the glass surface, it has a low surface charge; therefore, zwitterionic TMR is not

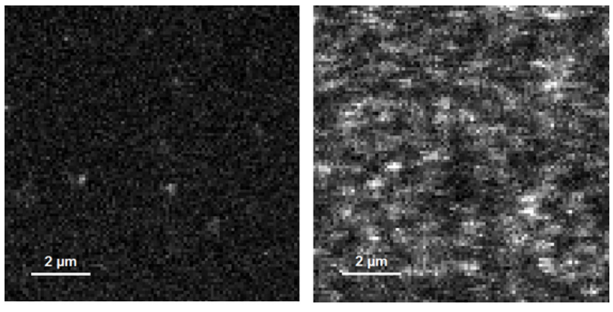

***Figure* 2.** Fluorescence images of surface adsorption of (a) TMR and (b) TMR biocytin by incubating TMR and TMR biocytin on a BSA-coated glass coverslip for 20 min.

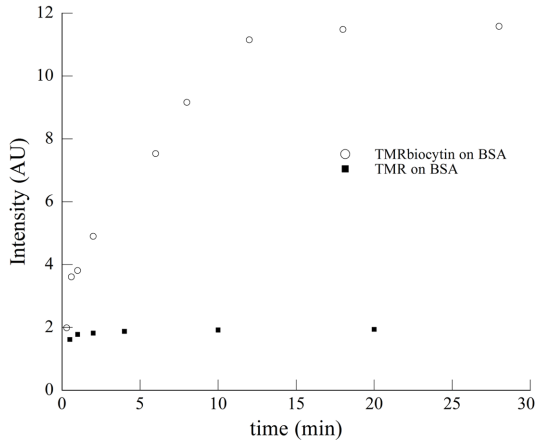

***Figure* 3.** Fluorescence intensities of TMR and TMR biocytin adsorbed onto the BSA surface over time.

effectively adsorbed. The degree of coverage was much higher than that of TMR, and this difference was due to the presence of biocytin. TMR biocytin contains three aldehyde groups, two tertiary amines, and one secondary amine group between TMR and biotin. These functional groups can form hydrogen bonds with the BSA surface. In addition, although the number of hydrophobic interactions between the biotin molecules and BSA was lower than that with streptavidin, hydrogen bonds are formed between the heteroatoms of the ureido ring and BSA.[14] Moreover, at pH 8.3, BSA adsorbed on the surface has a negative charge, and the amine group of TMRbiocytin has a positive charge due to the protonation of the three amine groups in biocytin, resulting in a strong electrostatic force between BSA and TMRbiocytin. Therefore, it is predicted that changes in the degree of adsorption can be observed by adjusting the pH. Figure 3 shows the fluorescence intensity of the TMR and TMR biocytin adsorbed on the BSA surface over time. The amount of TMR biocytin adsorbed onto the surface was measured by obtaining surface images scanned over a short period and averaging the photon signals stored in the image files. Photobleaching was minimized by maintaining a low intensity of the excitation laser and minimizing the retention time during scanning. As shown in the figure, TMR was adsorbed onto BSA-coated glass coverslips within 5 min and maintained the same coverage. This implies that after rapid adsorption to the site to be adsorbed, no further adsorption occurred. By contrast, the coverage rate of TMRbiocytin increased until approximately 12 min and remained constant thereafter. Therefore, the difference in the fluorescence intensity between the adsorbed TMR biocytin and TMR was owing to the presence of biocytin. In this experiment, the interaction or

surface adsorption between proteins and chemicals was investigated based on the fluorescence intensity after binding a fluorescent substance to a chemical substance. In this study, the interaction or surface adsorption between proteins and chemicals was investigated based on the fluorescence intensity after binding a fluorophore to a chemical. It is expected that proteins of interest and other chemicals will be studied similarly.

EXPERIMENTAL

A detailed description of the experimental setup can be found elsewhere.[15] Fluorescence lifetime imaging was obtained using a home-built microscope setup equipped with a 63x total internal reflection fluorescence (TIRF) objective (α Plan-Apochromat, 1.4. Zeiss). The wavelength of the excitation laser required to obtain fluorescence imaging was approximately 510 nm and the excitation was performed using a diode laser (PDL 800D, PicoQuant) with a pulse width of approximately 100 ps. The size of the excitation laser beam is expanded and directed to the objective lens through a dichroic mirror (Thorlabs, DMLP638) mounted on the microscope. Samples on the slide surface are excited by the focus generated through the objective lens, and the generated fluorescence is collected with the same objective lens and filtered through a band pass filter (FF02-510/10-25, Semrock) and a 75 μm diameter confocal pinhole (Thorlabs, P75H) to eliminate out-of-focus fluorescence. Fluorescence passing through the confocal pinhole was sent to an avalanche photodiode (APD), and the fluorescence signal was recorded using a time-correlated single photon counting board (Timeharp 260 PICO, PicoQuant). Symphotime64 (PicoQuant) was used to obtain fluorescence lifetime imaging and analyze the data. The cover slide used in the experiment had a thickness of 0.13 to 0.16 mm, and the surface was treated with a plasma cleaner. TMR and TMRbiocytin used in the experiment were purchased from Sigma, and BSA was also purchased from Sigma.

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