Single Molecule Tracking of Cholera-Toxin Subunit B on GM1-ganglioside Containing Lipid Bilayer*

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Diffusivity of Cholera toxin subunit B (CTX) bind to GM1 molecules in 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC) bilayer was observed by total internal reflection microscope (TIRFM). Numerical analysis based on mean square displacement revealed the presence of higher \( D = 0.4 \mu \text{m}^2/\text{s} \) and lower \( D < 0.1 \mu \text{m}^2/\text{s} \) diffusive fractions, both of which were an order of slower compared to dye-labeled lipid \( D = 3.5 \mu \text{m}^2/\text{s} \). The observed difference in CTX-GM1 diffusivity reflects the characteristics of the multivalent CTX-GM1 binding properties.

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I. INTRODUCTION

The lateral diffusion dynamics of bio-molecules in cell membrane is a key factor to determine their biological activity. Especially the formation of ligand–receptor binding in the cell membrane is an important phenomenon to reduce diffusivity giving confinement of the receptor to a preference site. Diverse kinds of biological events such as signal transduction are associated on the ligand–receptor binding. Bacterial toxins are also known to appear their activity through the binding to their receptor binding. Among them, cholerae GT (CTX), a pathologically active agent secreted by Vibrio cholerae, is known to form multivalent binding to the ganglioside GM1 [3]. The multivalent binding is supposed to strongly reduce the lateral mobility of the ligand CTX in the cell membrane. In spite of the importance of the diffusion behavior of these units, detailed information based on in-situ real time observation has not been obtained yet. Acquiring direct information on the change in the lateral mobility through the multivalent binding will yield deep insight into biological and physical understandings of the CTX-GM1 multivalent binding phenomena.

Very limited and averaged information has been reported for CTX-GM1 system, which were mainly examined by fluorescence microscope (FM), fluorescence correlation spectroscopy (FCS), atomic force microscopy (AFM) [4–10]. Although simultaneous measurement of AFM and FCS [4, 6] is very powerful to make clear the relationship between the molecular diffusivity and the bilayer phase, FCS measurement offers only averaged information on the diffusivity for several molecules existing in the probed area. Even if there are major and minor fractions, slightly different diffusivities makes difficult to distinguish them. Contribution from major fraction was often extracted as a single component. One may adapt direct observations of respective single molecule in the lipid bilayer, such as total internal reflection fluorescence microscopy (TIRFM), which can evaluate the diffusivity of each molecule in real-space [11]. In the present study, we have carried out TIRFM observation of diffusion behavior of CTX molecule binding to GM1 molecule in the lipid bilayer.

II. EXPERIMENTAL

1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC) (Avanti Polar Lipids), ganglioside GM1 from bovine brain (Sigma), CTX subunit B - Alexa Fluor 555 conjugate (we describe simply CTX hereafter) and Texas Red 1,2-dilhexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE) (Invitrogen), phosphate buffer solution (Wako) were used as a substrate for single molecule observation. The substrate was cleaned with water and acetone in an ultrasonic bath.Then the substrates were immersed in a piranha solution (70:30 v/v of concentrated H2SO4 and 30% H2O2; caution, the piranha solution reacts violently with organic materials and extreme care must be taken at all time when handling) for 10 min. After being rinsed with hot water, the substrates were dried with nitrogen flow.

The lipid bilayer was prepared by a self-spreading method according to a previous paper [12, 13]. Briefly, a small amount of chloform solution of DOPC/GM1 (99/1 mol ratio) was dropped on the substrate. After being dried in air, the substrate was immersed in a phosphate buffer solution. The bilayer spread on the substrate just after the immersion. Then, CTX buffer solution was added. The final CTX concentration was adjusted to be 10⁻¹¹ M. In the case of Texas Red DHPE, a chloform solution containing DOPC and Texas Red DHPE (10⁻⁵ mol%) was dropped, instead of DOPC/GM1 solution.

The single molecule observation for CTX and Texas Red DHPE was carried out with TIRFM. Our setup is illustrated in Fig. 1. In short, an excitation laser beam (532 nm, 10 mW) was delivered through an objective lens \((100 \times, \ N. \ A. = 1.45)\) and totally reflected at the substrate surface. Emitted photon from Alexa 555 moiety of CTX bound to GM1 was detected by an intensified CCD camera. The flame rate was 60 flames/s.

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Figure 1 shows typical diffusion trajectories of bright spots observed by TIRFM measurement. The observable duration was determined by the photobleaching-life time of each dye molecules. Typically, photobleaching was observed for 3 to 5 sec. During the duration, Texas Red DHPE demonstrated long diffusion over 50 μm. However, the diffusion length of CTX within a similar duration was found to be significantly small compared to Texas Red DHPE. Our control experiment, in which CTX was added DOPC bilayer not containing GM1, did not show distinctive bright spots. These results strongly suggest that CTX recognizes GM1 as a binding site. Observed bright spot at TIRFM observation are attributable to CTX-GM1 conjugate. In the present condition, the spreading distance for 5 sec is below 1 μm. Thus the effect of molecular drift due to the self-spreading can be neglected.

For further quantitative discussion on the diffusivity difference between Texas Red DHPE and CTX, we carried out mean square displacement (MSD) analysis. In the MSD analysis, the molecular diffusivity is characterized by diffusion coefficient \( D \).

\[
\langle r^2 \rangle = 4Dt, \tag{1}
\]

where \( t \) is the time interval. The results of MSD analysis for both Texas Red DHPE and CTX were shown in Fig. 3. In the case of Texas Red DHPE, \( \langle r^2 \rangle \) reached 50 μm² for 1 sec, whereas most CTX reached at most 10 μm². Only a minor fraction showed a similar diffusivity as Texas Red DHPE. The CTX molecules with higher diffusivity are though to be brought from (i) weakly bound monovalent CTX-GM1 or (ii) multivalent CTX-GM1 with directional motion. The latter is also a characteristic behavior of the Brownian motion that can be observed especially for a short time observation.

To evaluate \( D \) through Eq. (1), using \( \langle r^2 \rangle \) only for a first few steps is better to yield more reliable data [14]. Furthermore, the raw MSD data occasionally contains the spatial noise [15]. Therefore, one should subtract the spatial noise from MSD data for all step other than \( t = 0 \). One can also eliminate the effect of the spatial noise simply by excluding the first step for the MSD analysis. We adapted the latter method and \( D \) was then estimated for all MSD data in Fig. 3 by using \( \langle r^2 \rangle \) of second, third and forth steps.
The results were shown as a $D$ histogram in Fig. 4. Both Texas Red DHPE and CTX systems show Gaussian distribution, which reflects the molecular diffusion activated by a thermal energy. The averaged $D$ of Texas Red DHPE was $3.5\,\mu\text{m}^2/\text{sec}$. This value is almost the same as the previously reported value of $D\,\sim\,2.7\,\pm\,0.3\,\mu\text{m}^2/\text{sec}$ for DOPC bilayer on solid support [16]. On the other hand, the averaged $D$ for CTX (ca. $0.4\,\mu\text{m}^2/\text{sec}$) was an order of lower than for Texas Red DHPE. Furthermore, a fraction showing highly suppressed diffusion with lower $D$ below $0.1\,\mu\text{m}^2/\text{sec}$ was also recognized in the histogram. Although the histogram around this region is slightly obscure due to the insufficient sampling number. However, it should be noted here that CTX that is non-specifically bound to glass surface shows $D\sim\,0.01\,\mu\text{m}^2/\text{sec}$, which can be clearly distinguished from the MSD with $D\,=\,0.1\,\mu\text{m}^2/\text{sec}$. Thus, both fraction with $D\,=\,0.1\,\mu\text{m}^2/\text{sec}$ and $0.4\,\mu\text{m}^2/\text{sec}$ can be suggested to be brought from CTX that are on GM1-containing lipid bilayer. Relatively low $D$ for CTX would be due to the formation of multivalent binding with GM1, similar to the diffusion lowering observed for multivalent binding between lipid and gold nanoparticle [11]. It has been reported that CTX show concentration-dependent multivalent binding to GM1 [8]. Under the present condition, 1 mol% GM1 in the bilayer exposed to a solution containing $10^{-11}\text{ M}$ CTX, the binding equilibrium between CTX and GM1 can be explained by Hill-Waud binding model. This supports the formation of the multivalent binding in the present system. CTX is known to bind to at most five GM1 molecules in the cell membrane [3]. It is also known that the binding number is sensitively changed in the artificial bilayer depending on not only the concentrations of CTX and GM1 but also the lipid composition [5, 8]. Although the exact determination of the binding number in the present system is out of the object, any of binding number (one to five) would be involved in the present system.

Additionally, we found the presence two species with different $D$. As discussed above, the molecular diffusivity is related to the number of binding site on the lipid bilayer. Thus, we attributed these species as low- and high-valence conjugate of CTX-GM1. So far, FSC measurement has also been used for the determination of the diffusivity of CTX-GM1, and similar two-component results were obtained [4]. However, the fitting procedure for FCS curve needs great care for both experimental and analytical protocols to extract well-reliable data. Contrary to FCS, we can show that the direct observation and analysis of each single molecule would yield more reliable information. Our present result strongly supported the presence of high and low diffusive fraction in CTX-GM1 conjugate, reflecting the variation in the binding number. Since the binding between CTX and GM1 seems not to be static, it is probable to change the number of binding during observation. This kind of dynamic change will be detected as the change in the diffusivity during continuous observation by TIRFM, whereas FCS would detect as two independent molecules with different $D$. We are now trying to make clear this issue, and the results will be appeared elsewhere.

IV. CONCLUSIONS

We succeeded in a single molecule observation of CTX bound to GM1 in the lipid bilayer. MSD analysis made clear the present of two components with different diffusivity, both of which are an order of slower compared to Texas Red DHPE in the same condition. The difference in the molecular diffusivity in CTX-GM1 was attributable to the difference in the number of binding between CTX. Slower fraction seems to be brought from higher-valence CTX-GM1, due to higher apparent molecular volume via GM1-bridging structure in the lipid bilayer via CTX.

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