Single Molecular Motors

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Single-Molecule Imaging of Rotaxanes Immobilized on Glass Substrates: Observation of Rotary Movement**

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Biological molecular motors such as muscle fibers, flagella, and cilia[1–8] are precisely designed in nature, and are reminiscent of microscale electronic and mechanical devices.[9–10] Inspired by the direct observation of rotary molecular motor systems,[11] including F_oF_1-ATP synthase and F_1-ATPase, some research groups have attempted to directly observe artificial molecular motors.[12–16] For example, the three-dimensional orientation of myosin V was determined using bifunctional rhodamine as a probe.[17] Even smaller artificial molecular motors are extremely difficult to see, and thus, additional visualization techniques are necessary to observe rotational behavior. Rotaxanes have a suitable structure for the construction of molecular motors. We have previously investigated the rotational motion of a cyclodextrin relative to an axis molecule using NMR techniques.[18] We describe herein a single rotaxane molecule based on α-cyclodextrin (α-CD) functionalized with a fluorescence probe immobilized on a glass substrate, and estimate the rotary movement of α-CD by total internal reflection fluorescence microscopy (TIRFM).[19] When a single fluorescent molecule was observed by TIRFM, the luminescence spot measured about 150 nm. Even if an α-CD bearing a chromophore could show rotary movement centering around the molecular axis of an immobilized rotaxane, it would be difficult to directly observe the three-dimensional motion of the α-CD by conventional fluorescence microscopic techniques.[20] We have therefore employed the defocused wide-field imaging technique,[21–22] which functions by defocusing on a chromophore of about 1 μm. The orientation of the emission transition dipole of a single chromophoric molecule is determined by its characteristic emission intensity distribution. In this study we observe the rotary movement of the α-CD component of a rotaxane by measuring the emission dipole orientation of the chromophores.

To immobilize the fluorescent rotaxanes a glass substrate coated with 3-(triethoxysilyl)propyl isocyanate (ICTES) and *N*-propyltriethoxysilane (PTES) was functionalized with 4,4′-diaminodiphenylacetylene. This was accomplished by immersing the glass substrate first in a dimethylformamide (DMF; 10 mL) solution of the diamine and then in an aqueous solution of α-CD modified with rhodamine B (6-RhB-α-CD) to give the pseudorotaxane on a glass substrate (G-pseudo-R-CD, Figure 1). G-pseudo-R-CD was stoppered by immersion in a solution of 5-isothiocyanate isophthalic acid[23] to yield the rotaxane with 6-RhB-α-CD on a glass substrate (G-R-CD, Figure 1; the preparation is described in detail in the Experimental Section).[24] Before the glass substrate was examined by TIRFM, it was rinsed and sonicated in aq. HCl (pH 3.5), water, methanol, toluene, acetone, and methanol to remove free 6-RhB-α-CD.

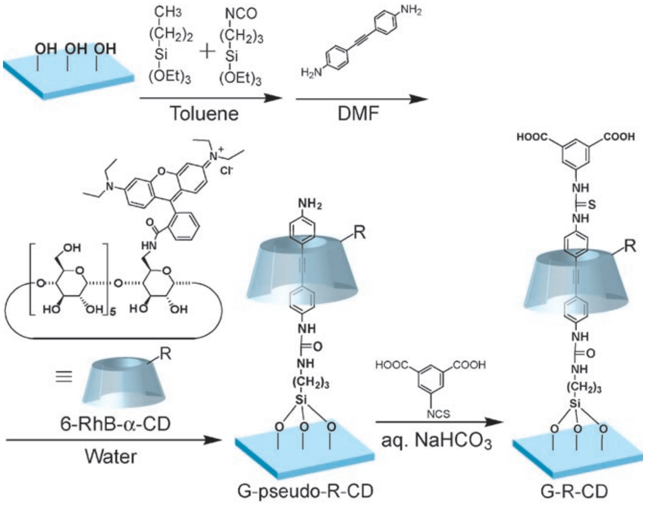

Figure 1. Schematic diagram of the preparation of rotaxane (R-CD) with rhodamine B-modified α-CD on a glass substrate.

When the nonfunctionalized glass substrate was immersed in an aqueous solution of 6-RhB-α-CD and subsequently washed, few luminescent spots were observed upon irradiation at 532 nm (Figure 2a), indicating that any 6-RhB-α-CD physically adsorbed on the glass substrate had been removed. When we used nonmodified α-CD instead of 6-RhB-α-CD for the formation of G-pseudo-R-CD, luminescent spots were not observed, indicating that 4,4′-diaminodiphenylacetylene

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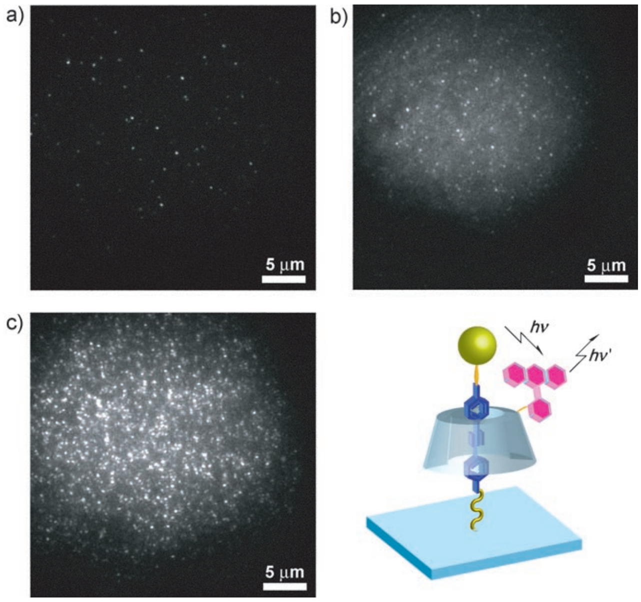

Figure 2. Fluorescence images of 6-RhB-α-CD (a), single pseudo-R-CD molecules and single R-CD molecules (b), and on glass substrates (c). The glass plates were illuminated with a Nd:YVO_4 laser at 532 nm with an excitation power of 3 $KW cm^{-2}$. Fluorescence emission was isolated from laser light by a dichroic mirror and a bandpass emission filter (550–610 nm), and imaged onto a cooled CCD camera. TIRF images were acquired with 100 ms integration times.

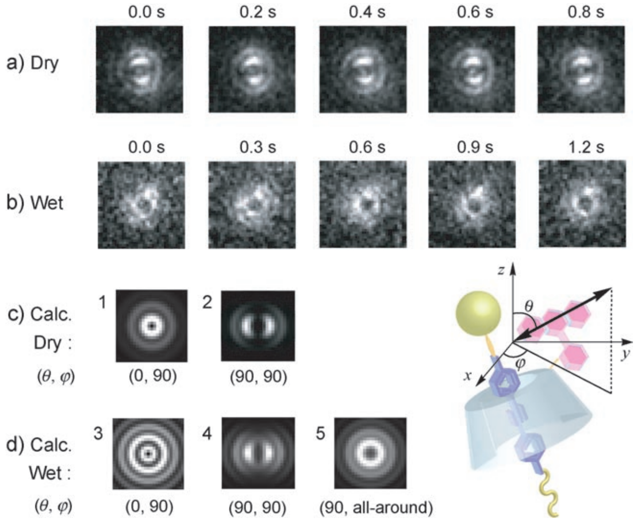

Figure 3. a) Snapshots of experimentally observed defocused images of a single R-CD molecule in the dry state over 0.8 s. b) Snapshots of experimentally observed defocused images of a single R-CD molecule in the wet state over 1.2 s. The contrast of these images was enhanced. The calculated emission patterns with variable parameters (θ, ϕ) are shown in (c) and (d): (0, 90) for images 1 and 3; (90, 90) for images 2 and 4. The averaged pattern for random orientation ($\theta = 90°$ and ϕ = all-around angle) is shown as image 5.

($\lambda_{max} = 430$ nm) acts soley as the rotor axis and is not responsible for luminescent spots when irradiated at 532 nm.

G-*pseudo*-R-CD showed fewer luminescence spots than G-R-CD (Figure 2 b, c). We hypothesized that washing removed most of 6-RhB-α-CD from the axis molecules of G-*pseudo*-R-CD on the glass substrate due to the absence of the stopper. In contrast, the rotaxane G-R-CD was stable on the glass substrate even after it had been rinsed and sonicated in certain solvents. The intensity and distribution of the fluorescent spots depended on the concentration of 6-RhB-α-CD, and, furthermore, each spot showed blinking and discrete bleaching, indicating single-molecule detection (Figure S1 in the Supporting Information).[25,26] These results indicate that stable rotaxanes with a fluorescent molecular probe are immobilized on the glass substrate; the observed luminescent spots are attributed to the fluorescence from the rhodamine B group of 6-RhB-α-CD.

To probe the orientation of the emission dipole of the chromophore, G-R-CD was measured using defocused imaging techniques under both sets of conditions. Figure 3 shows typical emission patterns of a single rotaxane molecule under dry and wet conditions. The emission of dry G-R-CD shows two bilaterally symmetric two-lobe patterns that do not change with time (Figure 3 a). The emission patterns in the wet state are different, revealing sets of concentric circles (Figure 3 b). The defocused pattern of a single dipole emitter can be theoretically calculated.[19] Figure 3 c,d shows the emission patterns calculated for different polar and azimuthal angles (θ and ϕ) of the transition dipole moment of rhodamine B. At $\theta = 90°$ and $\phi = 90°$, the calculated emission pattern showed a bilaterally symmetric two-lobe pattern. At $\theta = 0°$, the calculated emission pattern shows a symmetric circular ring. The defocused fluorescence image in the dry state fits well to the calculated pattern with variable parameters of θ and ϕ. For example, the molecule shown in Figure 3 a was evaluated to have the orientation of $\theta = 75°$ and $\phi = 9°$ at time $t = 0$. Thus, the three-dimensional orientation of individual molecules was determined by the defocused fluorescence imaging method. The rotational motion of a single molecule can be directly observed by the sequential acquisition of the defocused images. As shown in Figure 3 a, the orientation of the 6-RhB-α-CD remains constant at $\theta \approx 75°$ and $\phi \approx 10°$, indicating that the molecule is immovable in the dry state. Conversely, the circular defocused pattern in the wet state cannot be fitted to the calculation with the set values for θ and ϕ. Although the calculated image for $\theta = 0°$ also shows concentric rings, the predicted diameter of the inner circle is different from that observed. This result indicates that the emission dipole rotates within the time required for the image acquisition (300 ms). For comparison, image 5 in Figure 3 d was calculated by integration over the azimuthal angle from 0° to 360° (which assumes that 6-RhB-α-CD spins around an axis faster than the frame rate of the CCD), shows a pattern of concentric circles. This pattern corresponds to the experimental data in the wet state. We speculated that 6-RhB-α-CD swings around an axis and that the rate of the rotary movement of 6-RhB-α-CD is faster than 360°/300 ms.

In conclusion, the present study has addressed the rotary movement of 6-RhB-α-CD in a rotaxane structure adsorbed on a glass substrate. The rotary movement of 6-RhB-α-CD has been demonstrated by defocused wide-field imaging with TIRFM. Although TIRFM does not have a sufficiently high resolution to observe a substituent swinging on a rotor around a rotaxane, defocused imaging, which detects the transition dipole moment of a chromophore, is an excellent method for observing the rotational movement of a molecule. We have successfully observed fast rotary movement or rotary vibration in the wet state, and its investigation in the liquid or gel state is currently underway. Moreover, we are attempting to construct and directly observe unidirectional rotary motors.

Experimental Section

Preparation of derivitized surfaces.

Step A: Prior to monolayer formation, the glass substrates were ultrasonicated in toluene, acetone, and methanol for 15 min, respectively, and then dried with a stream of N_2. The substrates were subsequently cleaned with a UV-O_3 cleaner (UV253, Filgen) for 1 h to create a hydrophilic surface.

Step B: The cleaned substrates were immersed in a solution of PTES (2.18×10^{-3} mol), ICTES (1.48×10^{-7} mol) in toluene (10 mL) for 2 h at room temperature. The treated substrates were washed four times with toluene and quickly taken to next step.

Step C: The substrates were immersed in a solution of 4,4′-diaminodiphenylacetylene (5 mg) in DMF (10 mL) for 2 h at room temperature. These substrates were washed four times each with acetone and water.

Step D: The substrates were immersed in aqueous solutions (10 mL) of α-CD (1.02×10^{-4} mol) and 6-RhB-α-CD (5×10^{-9} mol) for 14 h at room temperature.

Step E: A solution of 5-isothiocyanateisophthalic acid (4 mg) and $NaHCO_3$ (3 mg) in water (1 mL) was added to the reaction solution and the solution was left for 5 h.

Step F: The substrates were rinsed ten times with water. Additionally, the substrates were rinsed four times and shaken for 15 min in the following solvents: aq. HCl (pH 3.5), water, methanol, toluene, acetone, and methanol. The substrates were then dried with a stream of N_2 and immediately imaged using TIRF microscopy (see the Supporting Information).

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