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Aggregation and fast diffusion of dye molecules on air–glycerol interface observed by confocal fluorescence microscopy

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Abstract

Diffusion processes of Rhodamine 6G (Rh6G) dye molecules dissolved in a small hemisphere drop of glycerol on a cover glass were investigated by using a confocal fluorescence microscope equipped with an objective lens with a high numerical aperture (NA = 1.35). Photon burst signals from Rh6G molecules in the bulk glycerol and on the air–glycerol interface of the hemisphere drop were separately detected at a single molecule level. The analysis of the photon burst signals by a correlation function method reveals that a sizable portion of the Rh6G molecules in the drop are aggregated on the air–glycerol interface and diffuse two-dimensionally on it, while the rest diffuse molecularly in the bulk. The aggregates are found to have a diffusion constant 15 times as large as that of the Rh6G molecule in the bulk glycerol, although the aggregates have a hydrodynamical radius much larger than that of a Rh6G molecule.

Keywords: Rhodamine 6G; Air–glycerol interface; Single molecule detection; Confocal fluorescence microscopy; Diffusion constant; Aggregation

1. Introduction

Glycerol has been a subject of considerable and long-standing scientific interest. The presence of three hydroxyl groups makes glycerol a particularly complex system as hydrogen-bonded fluids. In bulk glycerol, each glycerol molecule is bound by hydrogen bonds, of which formation and disruption are responsible to the high-viscosity of glycerol [1,2]. On the other hand, the hydroxyl groups of glycerol molecules on a glycerol interface tend to orient inward, while the CH and the CH_2 groups stick out of the interface [3]. The hydroxyl groups on the interface give rise to a high surface tension of the glycerol [4]. These findings indicate that solute molecules on the interface of a glycerol solution should behave differently from those in the bulk, because the interface and the bulk give totally different environments on the solute molecules.

As for other liquids, extensive studies have also been undertaken to elucidate the fundamental properties of the interfaces by taking advantage of various surface-sensitive tools [5–7]. In any case, molecules targeted for the investigation are needed to be abundant enough to attain an acceptable signal-to-noise ratio. In dealing with a small number of solute molecules targeted for investigation in a solution, fluorescence microscopy facilitates detection of weak fluorescence signals from the target molecules through measuring bunching signals of the fluorescence instead of measuring integrated dc signals of the fluorescence, although fluorescence spectroscopy is not well-compatible with this methodology. In addition, the fluorescence microscopy with a confocal configuration is particularly suitable for the investigation of dynamical behaviors of individual fluorescing molecules in a solution [8–11].

It is favorable from an experimental viewpoint that the objective lens of a fluorescence microscope employed has a numerical aperture larger than ∼1.3 so as to collect a suffi-

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cient number of fluorescence photons for the achievement of a single molecule detection. Note that the numerical aperture of the objective lens increases with the decrease of its focal length and hence the objective lens should have the shortest possible focal length. The sample and the micrometer should be so arranged that the objective lens is located at the closest possible distance to the sample.

In the present study, we prepared a hemispherical surface of a glycerol liquid containing a trace amount of Rhodamine 6G (Rh6G), which can be approached to the top of the objective lens at the distance of several tens micrometer, and investigated diffusion of a single Rh6G molecule in a bulk glycerol and its aggregate on an air–glycerol interface by using a confocal fluorescence microscope equipped with an objective lens of a large numerical aperture (NA = 1.35). In practice, the air–glycerol interface of the hemisphere drop of the Rh6G-containing glycerol solution on a cover glass was placed at a distance of several tens micrometer from the top of the objective lens by taking advantage of the fact that glycerol has a high surface tension. Bunching signals of fluorescence from Rh6G were measured by changing the position of the objective lens, with adjusting the focal point of an excitation laser either on the air–glycerol interface or the inside of the hemisphere drop.

2. Experimental

2.1. Materials and sample preparation

At first, a concentrated Rh6G solution in glycerol was prepared. Then, a working solution was prepared by serially diluting the solution with glycerol down to 2.5 nM, and was stored in a glass vial wrapped in an aluminum foil for prevention from photo-degradation. Commercially available glycerol (Kanto Chemical Co. Inc.) and Rh6G (Eastman Kodak Co. Inc.) were used without further purification.

2.2. Apparatus

The confocal fluorescence microscope system used consists of an inverted microscope (IX70, Olympus Inc.), a CW argon ion laser (177G, Spectra-Physics) for the excitation of Rh6G, and a silicon avalanche photodiode (Model SPCM, EG&G Canada, a quantum efficiency of ∼75% and a dark noise of ∼7 counts/s) as the detector of fluorescence. The inverted microscope is equipped with an oil immersion objective lens having a numerical aperture of 1.35 (UplanApo 100×). The argon ion laser was introduced into the objective lens after being reflected by a dichroic beam-splitter and was tightly focused onto a sample solution. Under irradiation of the argon ion laser at 488 nm, a Rh6G molecule in the focusing region (designated as focal region or focal volume) of the objective lens, in which the excitation laser is the most tightly focused, emits 10^6 to 10^8 photons/s by repeating absorption and emission cycles. The fluorescence photons were collected by the same objective lens for the detection by the silicon avalanche photodiode. In the present confocal system, photons coming from the outside of the focal volume were removed by a pinhole (50 μm in diameter) placed on the primary image plane. The dichroic beam-splitter and a single interference bandpass filter (BP545-580, Omega Optical Inc.) were used to remove the excitation laser light and the Rayleigh- and Raman-scattered light. Both the pinhole and the silicon avalanche photodiode detector were mounted on *xyz* transition stages for precise alignment. Photon signals were acquired by using a multichannel scalar (SR430, Stanford Research System) based on a personal computer.

2.3. Measurement

A 100 μL sample solution was dropped on a cover glass, and was wiped back and forth until it became hemisphere drops with diameters of several tens micrometers. The cover glass was approached to the objective lens as closely as possible. At the first place, the excitation laser beam was focused above the air–glycerol interface of a hemisphere drop, namely, the focal volume is located just above the interface of the drop. Then, the focal volume was lowered by ∼1 μm with moving the objective lens downward, and the fluorescence signals were measured. The same measurement was repeated by lowering the focal volume by another ∼1 μm and so on, so that the most appropriate location was discovered for collecting the fluorescence signals from a desired place (air–glycerol interface, inside, etc.) of the drop (see Fig. 1). The fluorescence was detected and counted with the gate time of 1.31 ms. One peaked signal collected in the gate time of 1.31 ms is designated as ‘fluorescence photon count’ whose height is measured as counts per 1.31 ms, while a bunch of fluorescence photon counts resulting from a fluorescing species (a single Rh6G molecule or its aggregate) traversing across the focal volume is designated as ‘photon burst’ consisting of a finite number of fluorescence photon counts varying with the trajectories of the fluorescing species together with the spatial intensity distribution of the excitation laser beam.

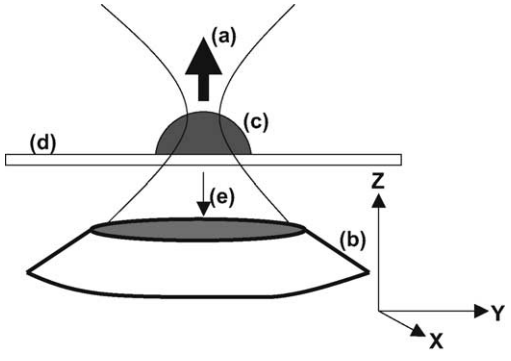

Fig. 1. Schematic diagram in the vicinity of the probe region of the apparatus employed: (a) excitation laser, (b) objective lens, (c) hemisphere drop of glycerol, (d) cover glass and (e) fluorescence.