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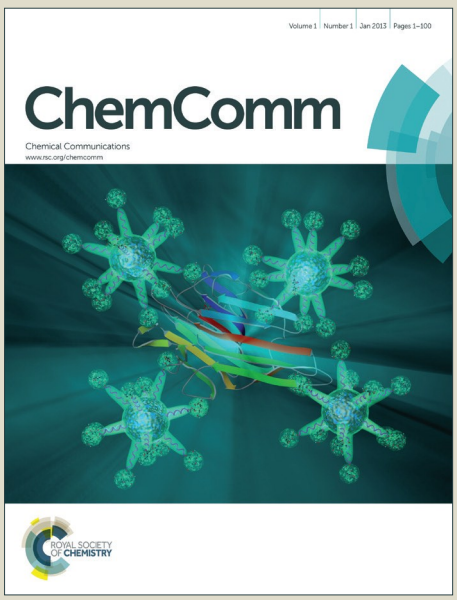

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Compact and stable SNAP-ligand conjugated quantum dots as a fluorescent probe for single-molecule imaging of dynein motor protein †

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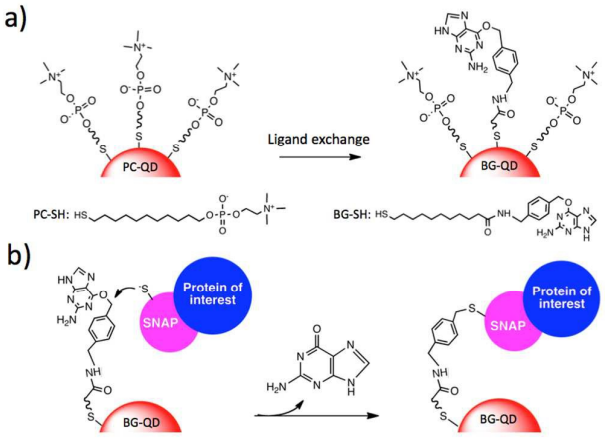
Compact SNAP-ligand conjugated quantum dots (< 10 nm) with high colloidal stability over a wide rage of pH (5-9) have been synthesized as a fluorescent probe for single-molecule imaging of dynein motor protein.

Single-molecule fluorescence imaging has provided exquisite details of the dynamics of various biological processes including membrane protein diffusion, motor protein kinetics, and DNA-protein interaction.[1] For such single-molecule imaging, very bright and robust fluorescent labels are needed, because photo-bleaching by excitation light obscures single-molecule tracking of the biomolecules. Semiconductor nanocrystals, called as quantum dots (QDs) are very bright and robust fluorescent materials compared to traditional organic dyes and fluorescent proteins.[2] In addition, QDs are suitable for multi-colored fluorescence imaging using a single-light excitation-source, because they have broad absorptions with narrow emission spectra.[2] These unique optical properties make QDs as ideal fluorescent probes for single-molecule imaging of biomolecules in living cells.[3] For the single-molecule imaging, smaller sized QDs with high stability are desirable to reduce the steric effects of QDs on the activities of biomolecules. Here, we report compact SNAP[4]-ligand conjugated QDs (< 10 nm) with high colloidal stability (pH=5-9) for single-molecule imaging of dynein motor protein. In this work, we have employed SNAP-tag technology to label the dynein motor protein with QDs. SNAP-tag is O^6-methylguanine DNA methyltransferase which can bind a benzylguanine (BG) moiety via a covalent bond.[5]

So far, there have been several types of protein tags such as GST-tag, His-tag and Halo-tag.[6-8] GST-tag[6] and His-tag[7] methodologies use the non-covalent binding with glutathione and nitroacetic acid in the presence of Ni^+ ions, respectively. Halo-tag method uses the covalent binding of Halo-tag fusion proteins with halo-alkane ligands.[8] SNAP-tag is one of the most used tag for the covalent binding of fluorescence probes to biomolecules.[9] Compared to halo-alkane ligands, SNAP-ligands are stable in aqueous solution because of the hydrophilic nature of BG moiety. Thus it was expected that the surface coating of QDs with SNAP-ligands will result in water-soluble QDs with high colloidal stability.[10] Although Fruk et al. first reported the preparation of SNAP-ligand conjugated CdSe/ZnS QDs, there is no report on the capability of SNAP-ligand conjugated QDs for fluorescence bioimaging.[11]



Scheme 1. a) Scheme for preparing SNAP-ligand conjugated QDs (BG-QDs) from PC-QDs by ligand-exchange. b) Binding reaction between a SNAP-tagged fusion protein and a BG-QD. Transfer of a benzyl group to the active site of SNAP-tag forms a covalent bond with the BG-QD.

As a fluorescent probe for dynein motor protein, we prepared SNAP-ligand conjugated CdSe/ZnS QDs (BG-QDs) from phosphatidylcholine (PC) coated QDs (PC-QDs)[12] using a ligand-exchange method as shown in Scheme 1. The PC-QDs have twitterionic phosphatidylcholine ligands at their surface and they are very stable in aqueous solution over a range of pH from 2 to 14.[13] To introduce BG-moieties to the surface of PC-QDs, BG-SH ligands ([BG-SH]/[PC-QD]=20) were conjugated to the PC-QDs (Scheme 1a, ESI). The binding mode of a BG-QD to a SNAP-tagged fusion

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protein is shown in Scheme 1b. A benzyl group in the BG-QD reacts with the cysteine molecule of SNAP-tag, and a guanine group is released to form a covalent bond between the BG-QD and SNAP-tag protein. The presence of BG-SH ligands on the QD surface was confirmed by MALDI-TOF mass spectroscopy (ESI).

The colloidal stability of BG-QDs in aqueous solution (10 mM PBS, pH=7.4) was examined by using fluorescence correlation spectroscopy. Fig. 1a shows a fluorescence autocorrelation curve of BG-QDs. For comparison, the fluorescence correlation curves of SNAP-tagged EGFP fusion protein (SNAP-GFP, 47.7 kDa, ESI) and Rhodamine 6G (Rh6G) are also shown. All fluorescence autocorrelation curves are fitted by a simple one-diffusion model,[13] where the diffusion times of BG-QDs, SNAP-GFP, and Rh6G are 0.35 ms, 0.25 ms, and 0.08 ms, respectively. Using the Stokes-Einstein relationship, the hydrodynamic diameter of BG-QD is estimated to be 1.4 times larger than that of SNAP-GFP.[14] The inset shows the pH dependence on the diffusion time of BG-QDs. The diffusion time of BG-QDs is almost constant (0.35 ms) over a range of pH (from 5 to 9), showing the high colloidal stability of BG-QDs. At the acidic condition at pH=4, the diffusion time of BG-QDs increased to be 0.7 ms, indicating the BG-QDs tend to aggregate at the low pH solution. The hydrodynamic size of BG-QDs was measured by dynamic light scattering. The diameter of BG-QDs was ca. 7.5 nm (Fig. 1b, ESI), and this size is two times smaller than that of BG functionalized QDs previously reported by Fruk et al.[11]

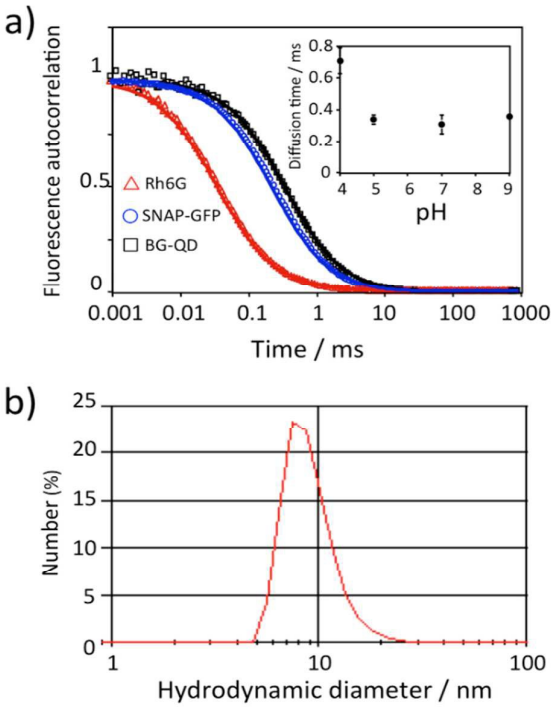

Fig. 1. a) Fluorescence correlation curves of Rh6G, SNAP-GFP and BG-QD in aqueous solution (10 mM PBS). The inset shows the diffusion times of BG-QDs versus pH. b) Distribution of hydrodynamic diameters of BG-QDs determined from dynamic light scattering experiments.

The binding ability of BG-QDs for SNAP-tagged proteins was checked by using a SNAP-GFP fusion protein. Fig. 2a shows the fluorescence correlation curves of BG-QDs in the absence and presence of SNAP-GFP. The addition of SNAP-GFP to the aqueous solution of BG-QDs ([BG-QDs]/[SNAP-GFP] = 5) increases the diffusion time of BG-QDs by a factor of 1. 5 fold (inset of Fig 2a). In contrast, the diffusion time of PC-QDs dose not change after the addition of SNAP-GFP (ESI). These results indicate that SNAP-GFP proteins specifically bind to BG-QDs. This binding between SNAP-GFP proteins and BG-QDs was confirmed by agarose gel electrophoresis (Fig. 2b) and size-exclusion chromatography (ESI). The mobility of BG-QDs+SNAP-GFP was smaller than that of BG-QDs, while the mobility PC-QDs+SNAP-GFP was almost same as that of PC-QDs. To evaluate the number of SNAP-GFP molecules binding to one BG-QD particle, we examined the fluorescence spectra of a complex of SNAP-GFP・BG-QD. The SNAP-GFP・BG-QD complex shows two fluorescence emission peaks resulting from GFP and QDs (Fig. 2c, ESI). Fluorescence life-time measurements indicated that the FRET from GFP to QDs dose not take place in this system[15] (ESI). Thus we can estimate the molecular number of SNAP-GFP binding to one QD particle from a calibration curve for the fluorescence intensity ratio of SNAP-GFP and BG-QDs (inset of Fig. 2c). The fluorescence intensity ratio of FI_{515}/FI_{600} in the spectra of SNAP-GFP・BG-QD complex is 0.23. The molecule number of SNAP-GFP binding to the surface of one BG-QD is estimated to be ca. 4.

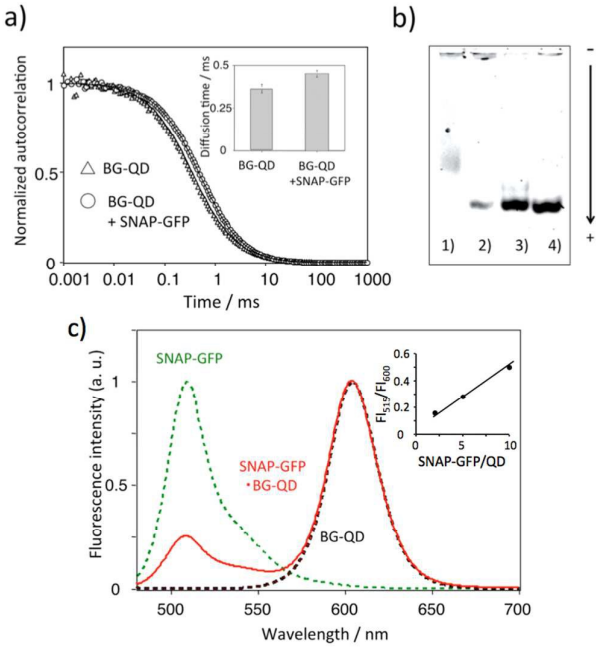

Fig. 2 a) Fluorescence correlation curves for BG-QD, and BG-QD + SNAP-GFP in 10 mM PBS. The inset shows the diffusion times of BG-QDs in the absence and presence of SNAP-GFP. b) Agarose gel electrophoresis of BG-QD + SNAP-GFP (1), BG-QD (2), PC-QD + SNAP-GFP (3), and PC-QD (4). Fluorescence was detected at > 600 nm. c) Fluorescence spectra of BG-QD, SNAP-GFP, and BG-QD・SNAP-GFP in 10 mM PBS. The inset shows a calibration curve of fluorescence intensity ratio (FI_{515}/FI_{600}) at 515 nm and 600 nm for the GFP emission in BG-QD + SNAP-GFP.

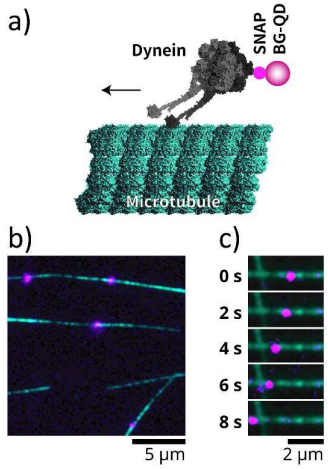

Fig. 3 Single-molecule imaging of BG-QD conjugated dynein. (a) Schematic representation showing the movement of BG-QD conjugated dynein along a microtubule. (b) Still image of QD-conjugated dynein (magenta) and microtubules (cyan). See also a movie in ESI. (c) Movement of a QD-conjugated dynein along a microtubule. In the presence of 1 mM ATP, the QD-conjugated dynein moved towards the left end of the microtubule.

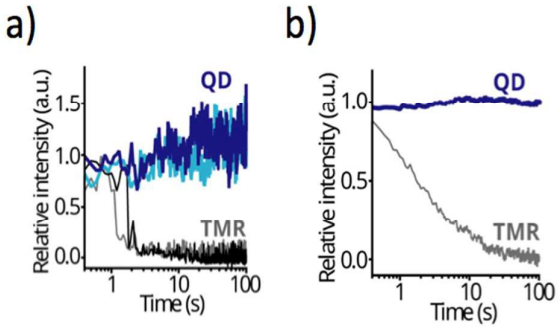

Fig. 4 (a) Time courses of fluorescence intensity of single BG-QD (blue and cyan) and single TMR (black and gray) molecules. (b) Time courses of the mean fluorescence intensity for 100 particles of QD and TMR.

To demonstrate the capability of BG-QDs, we performed single-molecule imaging of SNAP-tagged dynein protein *in vitro*. A BG-QD was conjugated to a SNAP-tagged dynein protein (Fig. 3a) and its single-molecule image was taken by using a total internal reflection microscope.[3] We observed fluorescence spots (magenta) of single BG-QD conjugated dynein molecules on a microtubule (cyan), where tubulin is labeled with Alexa Fluor 488 (Fig. 3b). In the presence of ATP (1 mM), a fluorescence spot (marked with an arrow) moves towards the left end of the microtubule (Fig. 3c). A movie of the single-molecule tracking clearly shows that a dynein molecule moves along a microtubule (movies in ESI). The robustness of BG-QDs was confirmed by a high-power excitation (~0.03 mW/μm^2) of a 552 nm laser. Time courses of fluorescence intensity are shown for single BG-QD molecules (Fig. 4a). For comparison, photobleaching data for tetramethylrhodamine (TMR) is also shown. TMR fluorescence shows single-step photobleaching within several seconds, while QD fluorescence is maintained. Fig 4b shows time courses of the mean intensity for hundred particles of QD and TMR particles. In the case of TMR, its photobleaching is ca. two seconds. In contrast, the mean fluorescence intensity of QDs is not decreased over 100-seconds illumination, showing that the BG-QDs are highly resistant to photobleaching even at the single-molecule level.

In conclusion, we have presented the synthesis of compact BG functionalized QDs as a single-molecule imaging probe. The hydrodynamic diameter of BG-QDs is less than 10 nm, and they are highly resistant to photobleaching. In addition, the BG-QDs are highly stable over the physiological pH from 5 to 9. We have demonstrated the capability of the BG-QDs for single-molecule imaging of dynein motor proteins along microtubules on a glass substrate. Although the BG-QDs in this study emit at visible region (ca. 600 nm), the emission of BG-QDs can be tuned to the second near-infrared (2nd-NIR) region (1000-1400 nm) using PbS QDs. [15b] Recent works[16] have demonstrated that the fluorescence imaging in 2nd-NIR region is a powerful tool for deep-tissue imaging in live animals. Second near-infrared emitting BG-QDs would make it possible to achieve *in vivo* single-molecule imaging of motor proteins in cells and tissues. The BG-QDs will be useful as fluorescent labels for SNAP-tagged biomolecules *in vitro* and *in vivo*.

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