This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Compact and stable SNAP-ligand conjugated quantum dots as a fluorescent probe for single-molecule imaging of dynein motor protein

Tatsuya Ohyangi a, Tomohiro Shima b, Yasushi Okada b, Yoshikazu Tsukasaki a, Akihito Komatsuzaki a, Setsuko Tsuboi c and Takashi Jin c *

Compact SNAP-ligand conjugated quantum dots (< 10 nm) with high colloidal stability over a wide range 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. 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. 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. These unique optical properties make QDs as ideal fluorescent probes for single-molecule imaging of biomolecules in living cells. 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-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 O6-methylguanine DNA methyltransferase which can bind a benzylguanine (BG) moiety via a covalent bond. So far, there have been several types of protein tags such as GST-tag, His-tag and Halo-tag. GST-tag and His-tag methodologies use the non-covalent binding with glutathione and nitroacetic acid in the presence of Ni2+ ions, respectively. Halo-tag method uses the covalent binding of Halo-tag fusion proteins with halo-alkane ligands. SNAP-tag is one of the most used tag for the covalent binding of fluorescence probes to biomolecules. 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. 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.

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) 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. 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-tag forms a covalent bond with the BG-QD.
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, \( I(t) = I_0 - \frac{2}{3}z^2 D t \), 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. \(^{11}\)

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 \( F_{615}/F_{600} \) 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. 1.](image1.png)

**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.

![Fig. 2.](image2.png)

**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 \( F_{615}/F_{600} \) at 515 nm and 600 nm for the GFP emission in BG-QD + SNAP-GFP.
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. Recent works 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.

T. J. thanks Ms. Miyuki Hasegawa for the synthesis of SNAP-GFP proteins. T. J. also thanks Prof. H. Yasuda and Dr. T. Sakata for measuring TEM images.

Notes and references

10 In the case of halo-alkane ligands, the colloidal stability of QDs significantly depends on the content of the halo-ligands presented on the surface of QDs. See ref 3).

15 The fluorescence lifetime of GFP emission in SNAP-GFP and SNAP-GFP・BG-QD was almost same (2.5-2.7 ns), indicating no FRET from GFP to BG-QD in the SNAP-GFP・BG-QD complex.