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Blinking effect and the use of quantum dots in single molecule spectroscopy

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ABSTRACT

Luminescent semiconductor nanocrystals (quantum dots, QD) have unique photo-physical properties: high photostability, brightness and narrow size-tunable fluorescence spectra. Due to their unique properties, QD-based single molecule studies have become increasingly more popular during the last years. However QDs show a strong blinking effect (random and intermittent light emission), which may limit their use in single molecule fluorescence studies. QD blinking has been widely studied and some hypotheses have been done to explain this effect. Here we summarise what is known about the blinking effect in QDs, how this phenomenon may affect single molecule studies and, on the other hand, how the "on"/"off" states can be exploited in diverse experimental settings. In addition, we present results showing that site-directed binding of QD to cysteine residues of proteins reduces the blinking effect. This option opens a new possibility of using QDs to study protein–protein interactions and dynamics by single molecule fluorescence without modifying the chemical composition of the solution or the QD surface.

1. Introduction

Because of their unique photophysical properties such as high photostability, brightness, broad excitation profiles, narrow size-tuneable fluorescence spectra (larger QDs have a greater spectrum shift towards red), fairly high quantum yield and very high molar absorption coefficients [1,2], luminescent semiconductor nanocrystals (quantum dots, QD) have emerged as promising tools for studying biological interactions and monitoring intracellular processes. QDs with hydrophilic shells have been used to construct a variety of QD-bioconjugates, which have found wide applications in biochemistry and biology, including imaging and sensing [3–8]. One of the major advantages of QDs is that they can be excited efficiently over the entire spectral range from their emission band to the ultraviolet. This enables the use of a single excitation wavelength for multiple QDs emitting in narrow spectral ranges. In addition, QDs are highly resistant to metabolic degradation. These advantages allow investigations of QD labelled biomolecules with long observation times and high excitation light intensities and therefore are promising tools for single molecule spectroscopy.

1.1. The blinking effect in Quantum Dots

In the last years, the uni-molecular studies are becoming more and more popular in biosensing [9], biological [10–14], and imaging studies [15–19]. The main property that makes QDs attractive for fluorescence-based assays is their stability and high sensitivity. Despite the above mentioned advantages, applications of QDs using single-molecule spectroscopy have been hardly reported [20–22]. This is mainly due to the strong blinking effect showed by QDs. i.e. an intermittence of fluorescence emission [23–25]. Whereas, this effect does not cause problems for their use in ensemble measurements, it limits application of QDs in single-molecule spectroscopy.

The blinking behaviour continues being an impediment for using QDs in single molecule measurements. The causes of this intermittent light emission remain unclear. Several experimental [25–39], and theoretical models [40–48], have been proposed in order to explain this phenomenon. Between all these models, the most widely accepted mechanism was described by Efros et al. in the late 90's [41]. This model suggests that the blinking effect is the consequence of long-lived electron traps, where on/off state changes are regulated by trapping and detrapping events governed by Auger processes [49]. Although some modifications to this model have been suggested [29,36,46], the Auger effect still prevailed

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until it was recently challenged by two independent groups [50,51].

An explanation to reconcile both models was recently provided by Galland et al. [52,53]. They showed that QDs presented two different types of blinking, the conventional one due to the charging and discharging of the QD (Auger mechanisms) and a second one in which changes in emission dynamics are not accompanied by changes in the emission intensity and, thus, is not explained by the Auger effect.

Despite the clear disadvantages of the blinking effect for single-molecule studies, this property has been exploited in different experimental approaches [54]. Localisation of QDs using blinking statistics has been shown to allow super-resolution imaging of diverse structures including living cells [55–57]. In addition, this effect has been used to determine absolute positions of closely spaced QDs [58]. Last but not least, the unique photophysical properties of QDs–dopamine conjugates [59], has allowed using the blinking effect to follow dopamine incorporation and processing in a neuroendocrine tumour cell line [60].

In single-molecule studies the blinking effect is clearly disadvantageous and, thus, several efforts have been pursued to reduce it. To this aim different experimental approaches, mainly based on either varying the wavelength and power of the laser [44,61], or modifying the QDs structure (surface or core) [62–67], has been described. The proposed methods include immobilizing QDs near silver island films [68], adding TiO_2 nanoparticles [65], coupling to silver nanoprisms [64], encapsulating QDs in a thin, thiol-containing polymer film [62], or passivating the QDs surface with thiol groups [63].

To explain the effect of these modifications on blinking effect, Fomenko et al. showed that it is possible to selectively control the chemical QD environmnent to increase the radiative pathways during electron–hole recombination emission, providing a tool to suppress the QDs blinking [69].

We have previously used a QD-based FRET model to study ATPase rotary mechanism by single-molecule fluorescence spectroscopy [20,21]. Surprisingly, the binding of conventional water-soluble QDs to the b-subunit of ATPase abrogated the blinking effect observed in free QDs. Our results were similar to those obtained by using conventional fluorescence probes [70], validating our model using QDs. As indicated, the presence of thiol groups in the QD surface seems to reduce blinking effect and QD was bound to the ATPase through a cysteine group introduced by site directed mutagenesis. In order to analyse whether intrinsic cysteine groups of proteins may affect the blinking effect, we have attached appropriate QDs to different proteins via cysteine residues and investigated whether QD showed reduced blinking under these conditions.

2. Material and methods

2.1. TIRFM measurements

All single-molecule measurements were performed at room temperature. For TIRFM with immobilized QD490 the laser beam (Kr ion laser, 476 nm, 50 mW, Coherent) was focused to an inverse objective (UPlanApo 60x, 1.20 W, Olympus) and fluorescence intensity was detected with a liquid nitrogen cooled CCD camera (Roper Scientific, Visitron Systems) after passing the dichroic mirror DCLP 485 nm and the filter HQ 516/60 nm (AHF, Germany).

2.2. Labelling of QD490 to proteins

The next proteins were used: F0F1–ATPsynthase, F1–ATPsynthase, bovine serum albumin (BSA), Aprotinin, Trypsin, Fetuin, Lysozyme and immunoglobulin G (IgG). CdSe/ZnS quantum dot QD490 with an amino-modified surface (T2 Evi Tag, Lake Placid Blue) was purchased from Evident Technologies. To bind QD490 to proteins the amino groups of the quantum dot (approximately 4–6 amino groups per QD, Lake Placid Blue, Evident Technologies) were derivatized with *N*-[β-maleimidopropyloxy]succinimide ester (BMPS, Pierce). QD490 (10 μM) in 100 mM MOPS/NaOH, pH 7.9, 200 μM $MgCl_2$ and 0.1% *n*-dodecylmaltoside (DDM, Glycon) was incubated with 0.1 mM BMPS for 120 min at room temperature. Unreacted BMPS was removed by gel filtration (Sephadex G-25 fine, Amersham Biosciences). In parallel, proteins were reduced with DTT (100 mM) at 0 °C for 60 min and unreacted DTT was removed by gel filtration (Sephadex G-25 fine). The reduced proteins were labelled with QD490-maleimide using a molar ratio of 1:1 in PBS at 0 °C for 240 min. Unreacted QD490 was removed by gel filtration (Sephadex G-50 fine) as previously described [20].

3. Results

In order to analyse whether binding of QDs to a protein cysteine residue reduces blinking effect we have chose the proteins indicated in Table 1, and a water-soluble CdSe/ZnS quantum dot, QD490. These QDs are functionalised with amino groups at the surface and have a fluorescence emission maximum at 490 nm. To bind QD490 covalently to proteins the amino groups of QD490 were first derivatized with BMPS giving QD490-maleimide. QD490-maleimide was then bound to a protein cysteine residue, rendering a fluorescence labelled protein that carried one covalently bound quantum dot (see Experimental section).

To investigate quantum dot blinking, QD490 and QD490-protein were immobilized on a microscope cover slide by adsorption at the glass surface. They were illuminated by evanescent field excitation through the edge of the objective with a krypton ion laser at 476 nm, using total internal reflection fluorescence microscopy (TIRFM). Images of beads (Fig. 1a), the QD490 alone (Fig. 1b) and QD490 coupled to ATPase (F_0F_1-QD490; Fig. 1c) were taken with a CCD camera. Each luminescent spot results from a single QD. When the fluorescence intensity time trace of individual QDs was measured a strong blinking effect was observed (Fig. 2). The "on–off" reaction indicates that the luminescent spot is due to the emission of a single quantum dot.

QD blinking was drastically reduced when it was bound to the proteins as can be seen in the fluorescence intensity time trace of the quantum dot shown in Fig. 2. These results indicate that, irrespectively of the protein used, binding of QD to a protein cysteine group is the reason for the drastic reduction of blinking of QD490. The most plausible explanation for our findings is that the presence of a thioether bond close to the QD reduces the number of electron traps (off states), increasing the radiative pathways as previously suggested by using propyl gallate [69]. Indeed, the functionalization of QDs with thiols or thiol-containing materials was previously shown to efficiently reduce blinking

Table 1
Proteins with a free cysteine labelled with a QD490 and their corresponding molecular mass.

Protein with a free cysteine	Molecular mass
EF_0F_1	580000
Bovine serum albumin (BSA)	66400
F_1	382000
Aprotinin	6511
Trypsin	22000
Fetuin	36000
Lysozyme	14307
Antibody	210000

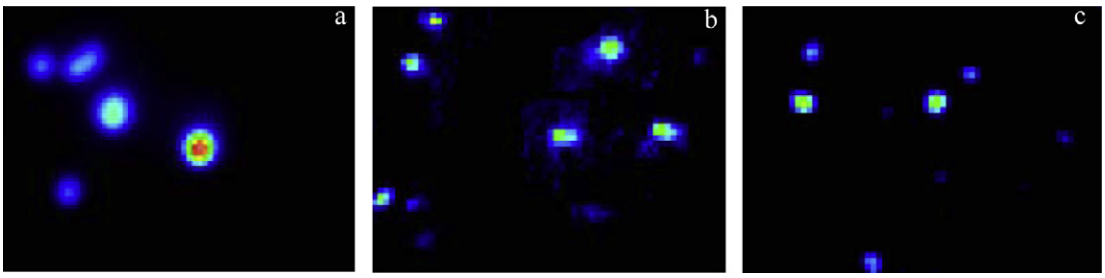

Fig. 1. Total internal reflection fluorescence microscopy (TIRFM) image of beads. (a) Water soluble quantum dots (QD490) and (b) QD490 bound to the H^+-ATPsynthase (QD490-F_0F_1) (c) Adsorbed at a microscope slide.

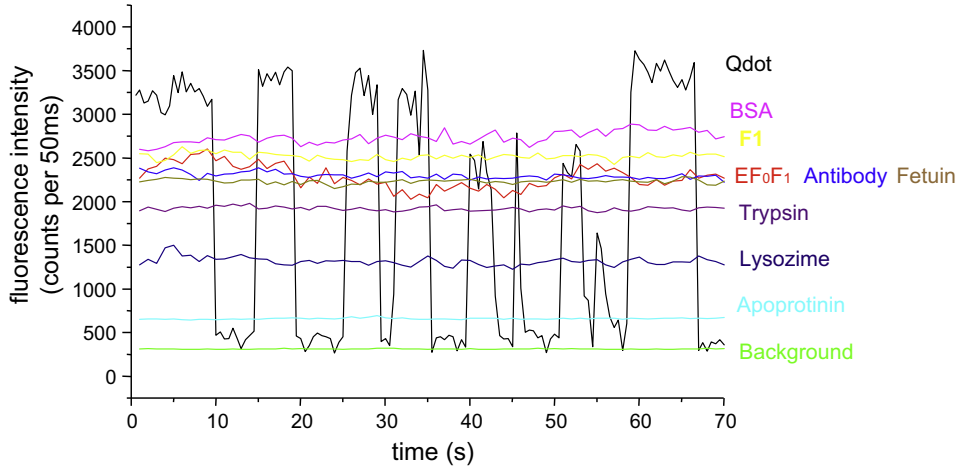

Fig. 2. Fluorescence intensity time traces of: QD derivatized with different proteins via a cysteine: EF_0F_1, bovine serum albumin, F_1, aprotinin, trypsin, fetuin, antibody and lysozyme.

[62,63,71]. The mechanism involved in this process was dependent on the ability of thiol groups to transfer electron into the hole traps of QDs [72]. Although the ability of thioether group to provide electrons is lower than that of thiols, it has been shown that the presence of specific functional groups in the vicinity of the thioether group highly increases the reducing potential of this group [73].

In order to analyse the effect of protein and/or crosslinker BMPS on the fluorescence stability of QD, the luminescence lifetime decays of QD490, QD490-BMPS and QD490-F_0F_1 were measured. As shown in Fig. 3, the presence of the BMPS crosslinker did not affect the decay rate of QD. However, the presence of ATPase clearly decreased decay rates, indicating that binding to proteins by an intrinsic cysteine group not only reduces the blinking effect but also increases the fluorescence life-time of QDs.

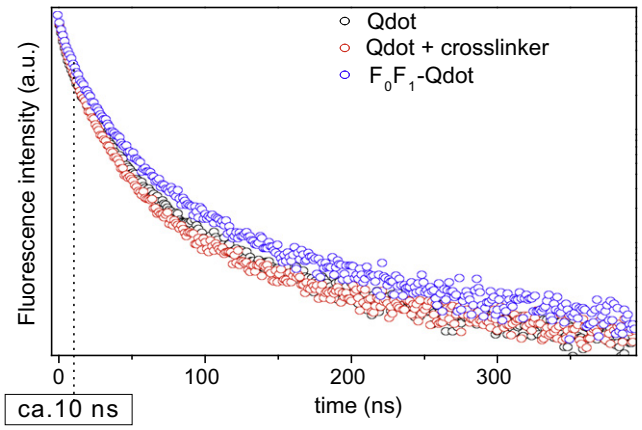

Fig. 3. Lifetime from QD, QD-BMPS and QD-F_0F_1.

4. Discussion

QDs have unique physico–chemical properties including high brightness and high photostability, making them a potent tool for single molecule studies of biochemical and biological processes. However their fluorescence emission is not continuous as they show a strong blinking effect, which limits their applications to study molecules at the single level. Here we have shown that it is possible to eliminate the blinking effect by binding of a water-soluble QD to a protein cysteine residue. This method relies on the presence of free cysteine groups. Thus, modification of proteins in which free cysteine groups only appear after reduction of disulphide bonds, will be subject to the relative importance of those bonds for protein function. In this case, introduction of a cysteine group by site-directed mutagenesis is highly advisable. This step is almost mandatory for studies in which QD must be bound to a specific protein location like during FRET-based analyses of protein–protein interaction and dynamics.

Our novel observation provides a fast, simple and direct method to study protein function and dynamics at the single level without further modification of solution or QD composition. Moreover, it could be applied to study any macromolecule where a free cysteine group is available.

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