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Photochemical & Photobiological Sciences

Cite this: DOI: 10.1039/c2pp25174d

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COMMUNICATION

NIR fluorescent biotinylated cyanine dye: optical properties and combination with quantum dots as a potential sensing device†

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Received 4th June 2012, Accepted 27th August 2012
DOI: 10.1039/c2pp25174d

Downloaded by McGill University on 30 October 2012
Published on 29 August 2012 on http://pubs.rsc.org | doi:10.1039/C2PP25174D

We present a water soluble and fluorescent biotinylated probe derived from a carbocyanine dye. A high efficiency of energy transfer was measured when the dyes were placed on the surface of streptavidin conjugated quantum dots. The system is a model platform for potential application as a FRET-based fluorescent sensor.

The biotin–(strept)avidin technology has been used for many years in a variety of different applications, especially in biotechnological techniques and assays.[1,2] The remarkable affinity for biotin ($K_a \sim 10^{15}$ M^{-1}) of egg-white protein avidin or its bacterial analog streptavidin has led to a large number of biotin-derivatives for *in vitro* and *in vivo* applications. Fluorescent derivatives of biotin have proven to be useful for indirect labeling of a biotinylated target (DNA, peptide, protein) by means of a bridge through streptavidin (SAv).[3–5] Such a probe for biomolecules must fulfil certain requirements for studies over a prolonged period of time: similar affinity as unaltered biotin, good water-solubility, high values of fluorescence efficiency in aqueous solution, and high photostability.

On the other hand, cyanine dyes (Cy), especially carbocyanines with optical properties in the near-infrared (NIR) spectral range, are known as outstanding labels for *in vivo* imaging,[6,7] because the relative contributions of autofluorescence of the sample and scattered light can be significantly reduced.

In the context of a project involving the synthesis of bifunctional carbocyanines, we found it worthwhile to generate biotinylated derivatives of cyanine dyes and to study their properties in a model FRET-based sensing system involving quantum dots (QDs) as donors.

The unique photophysical properties of these semiconductor nanoparticles are well known: broad absorption spectra, high quantum yields, reduced photobleaching and narrow emission spectra. Since the prototype sensor based on QD–protein–dye using MBP (maltose binding protein) placed on the surface of quantum dots,[8] many other examples have been reported combining different modifications on the QDs and a variety of analytes.[9]

We have investigated the performance of a dyad consisting of a water-soluble biotinylated carbocyanine dye (Fig. 1, CyBiot) and commercially available quantum dots conjugated to streptavidin. The system QD–CyBiot constitutes a donor–acceptor pair of particular interest in sensing studies based on FRET measurements by means of competitive inhibition or displacement assays in aqueous media. Very recently Achilefu *et al.* reported on the modulation of the emission of QDs by NIR carbocyanines as a strategy for pH-sensing nanomaterials.[10] Other available molecular dyes have functional limitations such as pH dependence, susceptibility to photobleaching and narrow excitation bands, all of which can compromise sensor performance. The use of cyanine dyes overcomes most of these limitations.

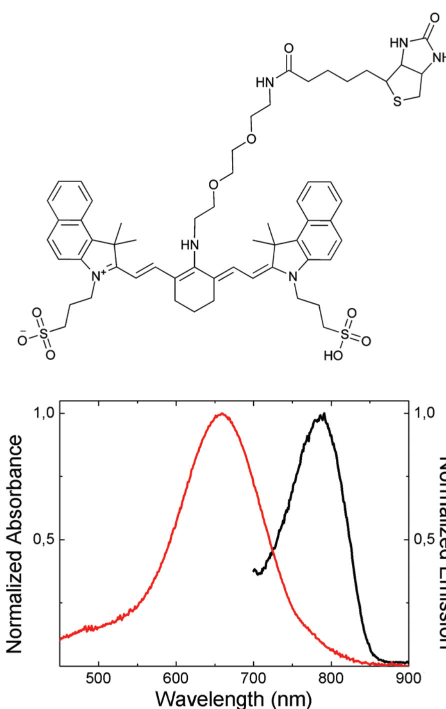

Fig. 1 Biotinylated cyanine dye CyBiot.

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† Electronic supplementary information (ESI) available: Detailed experimental procedures, characterization data, spectral properties, pK_a measurements, titration experiments. See DOI: 10.1039/c2pp25174d

‡ The corresponding author Elizabeth A. Jares-Erijman passed away during the revisions of this communication. The surviving authors dedicate it to her memory and in acknowledgment of her passion for life and work, inspired leadership and unfailing support.

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The synthesis of the biotinylated cyanine derivative CyBiot was performed from 1,1,2-trimethylbenz[*e*]indole, cyclohexanone and 1,3-propanesultone as the starting material, with modifications made on published procedures[11] (Fig. S1†). The presence of the chlorocyclohexenyl ring provides an additional site for further functionalization[12] while the sulfonyl groups increase the water solubility and biocompatibility. A chloro-substituted precursor was coupled with commercially available biotin–PEG–NH_2 to yield the biotinylated derivative. A major problem when following reported synthetic methodologies was the low yield obtained after purification of CyBiot. We optimized the purification procedure by using Sephadex LH-20 as the chromatographic phase instead of normal silica gel or reverse phase (RP-18). CyBiot is a deep blue solid with an absorption maximum at 660 nm (33 600 M^{-1} cm^{-1}) and emission at 790 nm (exc. 660 nm). This strong blue shift and large Stokes shift (>130 nm) are in agreement with previously reported aminocyanines.[6] The pK_a value of CyBiot was determined to be 5.0 ± 0.1 (see ESI†), ensuring pH insensitivity for emission fluorescence under the conditions used in the conjugation experiments.

The specificity of CyBiot was characterized by binding to streptavidin coated magnetic beads (Dynabeads M-280, 2.8 μm, Invitrogen). The beads were incubated with the biotinylated carbocyanine following magnetic separation and washing. The beads were then placed in microwell slides and examined with a wide field fluorescence microscope (Fig. S7†). As a negative control, another batch of beads was pre-incubated with biotin prior to the incubation with CyBiot. The image showed no fluorescence in the control sample, certifying the lack of non-specific interactions of the dye with the protein.

Most fluorescent biotin derivatives exhibit quenching upon binding to streptavidin. However, in the case of some cyanine derivatives the fluorescence was unaltered or even enhanced after binding.[3,13,14] The emission of CyBiot upon binding to SAv showed a moderate degree of quenching in the presence of the protein after successive additions at 3 min intervals (Fig. S4†). In control experiments, the protein was omitted before titration. The fluorescence quenching of the conjugates in 4 : 1 (dye–SAv) complexes was 40%, half the value observed for commercially available biotin-4-fluorescein.[15]

Conjugated QD655–SAv and biotinylated carbocyanine have a very favorable spectral overlap, leading to a large value of J (5.04 × 10^{17} M^{-1} cm^{-1} nm^4) (Fig. S6†). We calculated a value of R_0 = 13.0 nm, by using the parameters φ_D = 0.52; κ^2 = 2/3 (according to the general simplification, the proper value of κ^2 for QDs remains to be established); and n = 1.33 (corresponding to H_2O). This value of R_0 is bigger than that reported for other QD–dye systems (typically 5–6 nm).[16,17]

To study energy transfer between CyBiot (acceptor) and QD655–SAv (donor), we used two types of QDs provided by Invitrogen: ITK and poly(ethylene glycol) (PEG) conjugated QDs.

We chose 400 nm as the excitation wavelength, corresponding to a minimum in the excitation spectrum of the dye, thus minimizing direct excitation. As can be seen from Fig. 2, a substantial reduction in the emission of QD655 was observed after addition of increasing amounts of CyBiot, for both types of QDs. The efficiency of FRET was calculated according to the Förster theory.[18] In the case of ITK QDs the quenching was 90% at a ratio dye–QD of 100 : 1 and a quenching of 89% for the emission of PEG QDs for a ratio dye–QD of 34 (Fig. 2). The presence of SAv on the surface increases by ~5 nm the distance between the QD and the acceptor dye in comparison to a direct covalent attachment of the dye to the QD's surface[19] but this is compensated by the binding of multiple molecules of the acceptor to the surface, yielding a high efficiency of quenching.

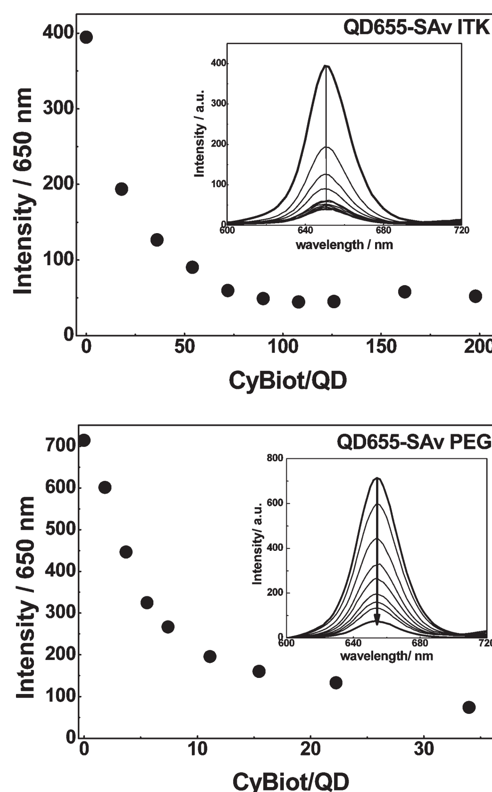

Fig. 2 Steady-state quenching of the emission of QD655–SAv ITK (upper panel) and QD655–SAv PEG (lower panel) by cumulative addition of CyBiot. λ_{exc} = 400 nm.

According to the manufacturer the differences between the QDs ITK and PEG conjugates reside in the attachment of streptavidin molecules to the surface of the nanoparticle. In the QD–PEG conjugate the protein is coupled to the amphiphilic polymer coating *via* a functionalized polyethylene glycol linker, in order to reduce the nonspecific binding, whereas in the ITK conjugate the streptavidin is directly attached to the inner amphiphilic coating without a PEG linker. The manufacturer reports that the ITK formulation results in an increase in the number of streptavidin molecules per nanoparticle relative to PEG-linked conjugates, which was confirmed in the literature.[20] Moreover, the titration curves of Fig. 2 and 3 are in good agreement with presumptions, regarding *ca.* 100 and 20 binding sites for ITK and PEG conjugates, respectively.

Nevertheless, our results show that the QD655–PEG performs a strong quenching by CyBiot than QD655–ITK, which could be counterintuitive. The manufacturer claims that the spectral properties of both systems are identical, therefore, the differences should be encountered in the distribution of the protein

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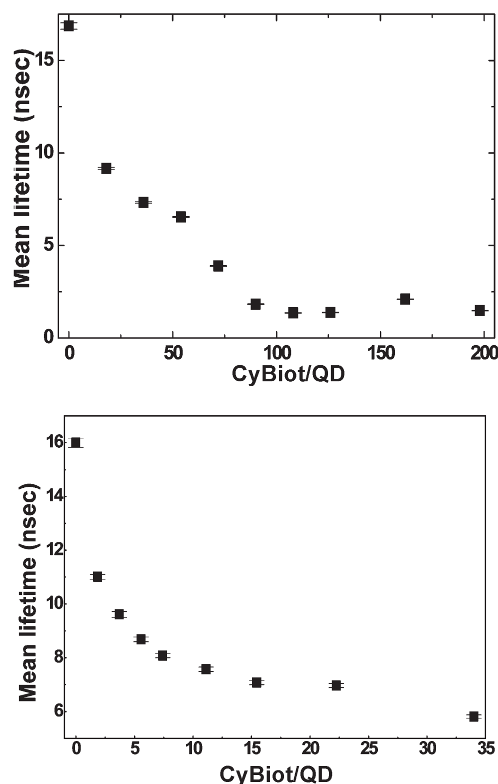

Fig. 3 Time-resolved quenching. Variation in the amplitude-weighted lifetime of the conjugates as a function of the [CyBiot] : [QD655] ratio. QD655–SAv ITK (upper panel) and QD655–SAv PEG (lower panel).

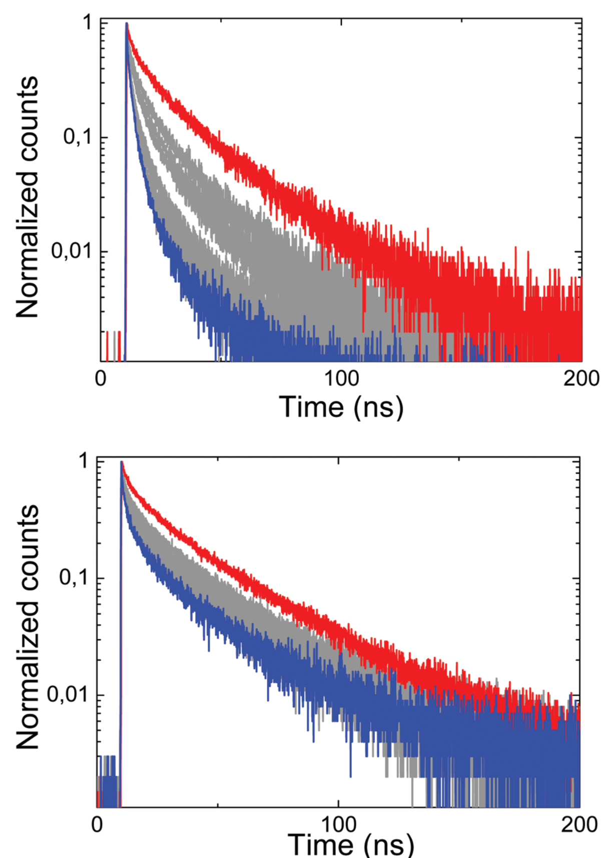

Fig. 4 Fluorescence decays for QD655–streptavidin conjugate titrated with CyBiot (initial, red line; final, blue line). QDITK (upper panel); QDPEG (lower panel).

molecules in the surface of both kinds of nanoparticles. Two models could be proposed: (i) all the binding sites in the ITK nanoparticles render a spatial orientation for CyBiot which reduces their FRET efficiency; (ii) a heterogeneous population model, all with the same affinity for the binding sites but different and distinguishable transfer efficacy, which reduces the overall FRET efficiency of the system.[21]

To further elucidate the energy transfer mechanism we performed the experiment by means of time-resolved techniques. The changes in the fluorescence lifetime of QDs were followed after titration with CyBiot (Fig. 3 and 4). Every titration point was fitted to a tri-exponential function, Table 1 shows the values of amplitude-averaged lifetime (τ_{mean}) for samples in the absence and with a saturating amount of CyBiot. The computed efficiency of FRET based on time-resolved techniques was 91% and 63%, for ITK and PEG QDs, respectively. Determinations of FRET efficiencies from lifetime measurements are less prone to errors due to aggregation and insolubility than steady state measurements, and are also less affected by scattered light. Lifetime measurements have the additional advantage of a reduced influence of the donor concentration. This issue is especially sensitive in our case because we worked with commercial samples with uncertainties in their nominal concentration. In an article from Grecco *et al.* some numeric models are discussed in order to explain this discrepancy between the values of FRET efficiency estimated by steady-state and time-resolved techniques for similar systems of QD–dye.[16]

Table 1 Time-resolved measurements (τ_{mean}) for QD655–SAv in the absence/presence of CyBiot

	Absence of CyBiot	Presence of CyBiot
QD655–SAv ITK	16.8 ± 0.2 ns	1.48 ± 0.01 ns
QD655–SAv PEG	16.0 ± 0.2 ns	5.90 ± 0.06 ns

In conclusion, the biotinylated probe described in this report is a potential excellent dye for indirect labeling strategies of biotinylated biomolecules in the NIR region. CyBiot showed a moderate degree of quenching binding to SAv compared to other biotinylated dyes, with no significant non-specific binding, and a high Stokes shift (130 nm) leading to a substantial reduction of potential *homo*FRET processes of the dye in a confined environment.

We have demonstrated that upon binding to the surface of streptavidin conjugated QDs, strong quenching of the emission of the nanoparticle occurs as a consequence of a FRET process.

As the surface of the QDs can be modified with other types of proteins and the carbocyanine structure has a great versatility regarding derivatization of its structure, this detection platform can be extended to other receptor–ligand systems for homogeneous competitive or inhibition assays.

Acknowledgements

We thank Dr Thomas Jovin for suggestions on the manuscript. The authors are grateful to ANPCyT (Argentina), CONICET (Argentina), University of Buenos Aires and Max Planck Society for financial support. G.O.M. is also grateful to CONICET for a PhD fellowship.

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