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The reversible modulation of the emission of CdSe/ZnS semiconductor nanocrystals (quantum dots) was achieved by binding photochromic diheteroarylethenes and switchable acceptors for fluorescence resonance energy transfer. A biotinylated diheteroarylethene derivative was bound to quantum dots bearing conjugated streptavidin, leading to an intensity decrease as a consequence of energy transfer to the closed form of the acceptor. Interconversion between the open and closed forms by irradiation with 365 and 546 nm light enabled deactivation and activation, respectively, of the FRET process with a corresponding modulation of quantum dot emission, observed both in solution and by sequential wide-field imaging.

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INTRODUCTION

As a consequence of their unique properties (non-toxicity, excitability by one of more photons over a wide spectral range, emission in a narrow and programmable spectral range, and above all, photostability) bioconjugated semiconductor quantum dots (QDs) are replacing organic molecules as fluorescence probes and sensors in numerous cell biological applications [1]. In bio-nanoelectronics, a field that encompasses the integration of biomolecules with novel nanomaterial components to create optoelectronic devices, light-driven devices are of central importance inasmuch as the wavelength, intensity, and exposure to light can be readily controlled. Biological molecules are attractive building blocks for such complex functional elements, the applications of which extend from nanosensing and nanocircuitry to computational memory. Quantum dots constitute an ideal component in this scheme.

We have recently introduced the use of photochromic compounds as switchable acceptors for fluorescence resonance energy transfer (FRET) determinations according to a scheme we denote as photochromic FRET (pcFRET) [2–7].

We have demonstrated that commercially available QDs bioconjugated to streptavidin and titrated with biotinylated fluorophores with suitable spectral properties are quenched efficiently by FRET (H. Grecco et al., manuscript in preparation). Others have reported a similar phenomenon using QDs conjugated with maltose binding protein [8]. This approach was extended by the same two groups by the incorporation of pc acceptors ([9], this work). While the cited publication [9] utilized a spiropyran-merocyanine photochromic system, we have favored the use of diheteroarylethenes due to the far greater thermal stability of their closed form, greater fatigue resistance, absence of solvent-driven dark degradation reactions, and greater compatibility with aqueous biological systems. These properties have enabled the use of QD-pcFRET in an imaging context, as featured for the first time in this communication.

EXPERIMENTAL

Chemicals

Biotin cadaverine **1** was from Molecular Probes (Eugene, OR). N, N′-dicyclohexyl-carbodiimide and N-hydroxysuccinimide were purchased

from Fluka Chemie (Buchs, Switzerland). Qdot-565-streptavidin conjugate QDs were from Quantum Dot Corp. (Hayward, CA). Compound **2** was reported previously [4]. We also refer to substructure **1** as Biotin and substructure **2** as DHE.

(3-(4-{4-[3,3,4,4,5,5-Hexafluoro-2-(2-methoxy-benzo[*b*]-thiophen-3-yl)-cyclopent-1-enyl]-3,5-dimethyl-thiophen-2-yl}-benzoylamino)-propionic Acid 2,5-dioxo-pyrrolidin-1-yl Ester (3)

10 mg (0.016 mmol) of **2** were reacted with 4 mg (0.020 mmol) of DCC and 2 mg (0.017 mmol) of N-hydroxysuccinimide in 3 ml of dry acetonitrile at room temperature for 2 h. The dicyclohexylurea was filtered and **3** evaporated *in vacuo*.

Compound 4 (Fig. 1b)

16 mg of biotin-longchain amine (Biotinyl-3, 6, 9-trioxaundecanediamine, Pierce EZ-Link Biotin-PEO-LC-Amine) (**1**, 32 μmol) were dissolved in 100 μl of dry acetonitrile and reacted with 32 μmol of

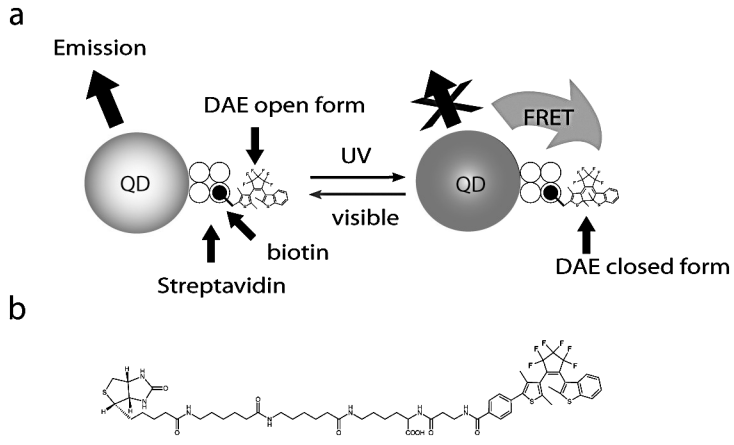

FIGURE 1 Schematic of pcFRET devices based on quantum dots. (a) Qdot-565-streptavidin QDs with bound biotinylated diheteroarilethene (DHE). Upon irradiation with UV light, the DHE, initially in the open colorless form, is converted to the closed colored form that functions as an FRET acceptor. Irradiation with visible light drives the DHE back to the open form, thereby deactivating the FRET process. In this manner, the emission properties of the QDs can be modulated by pcFRET. (b) Structure of the biotinylated diheteroarylethene, compound **4**.

compound **3** dissolved in 200 µl dry acetonitrile for 12 h at room temperature in the presence of 10 µl of triethylamine. The product (a white precipitate) was filtered and characterized by ^{1}H-NMR and ESI-LR and HR Mass spectrometry (ESIMS: M + H: 1204.4, HRESI: 1204.4550 , C58H72O8F6N7S3 (Δm: 3.4 mmu)

Steady-state Spectroscopy

Steady-state fluorescence measurements were performed with an SLM 8000S spectrofluorimeter at room temperature. The light source for photochromic conversion was a focused fiber-optic coupled 200 W Hg arc lamp with a UV bandpass filter (320–380 nm, output irradiance 3.5 mW cm^{-2}) for the photochromic forward conversion and a green filter (510–580 nm, output irradiance 20 mW cm^{-2}) for the backward conversion.

Microscopy

Imaging was performed on an Olympus IX71 inverted fluorescence microscope, equipped with a dual illumination system: (1) Hg arc lamp and filter selector (365 ± 10 nm, focal plane power and irradiance: 0.13 mW, 0.09 W cm^{-2}; 546 ± 10 nm, focal plane power and irradiance: 11 mW, 8 W cm^{-2}), and, alternatively, (2) a Xenon arc lamp source + P130 Optoscan monochromator (450 ± 15 nm, focal plane power and irradiance: 0.4 mW, 0.03 W cm^{-2}). Epi-illumination was with a 570 dichroic filter and the emission was collected with a 565 ± 20 nm filter optimized for the Qdot-565-streptavidin conjugate QDs. The 60 × 1.45 NA oil immersion objective had a field of view of 0.4 mm. Images (0.5 s exposure) were acquired with a CCD camera at an em gain of 50. Time series of 60 images (0.5 s exposures, 10 s intervals) were acquired during irradiation at 365 and 546 nm. They were interleaved with single images excited at 450 nm. Samples were prepared on poly-L-lysine pre-coated 18 well-flat µ-slides.

FRET Methods

R_o, the critical Förster distance (for 50% transfer efficiency), is defined by $R_o^6 = 8.785 \cdot 10^{-5}\kappa^2\Phi_D J n^{-4}$units, nm^6, where Φ_D is the quantum yield of the donor in the absence of acceptor, n is the refractive index of the medium, κ^2 is the orientation factor between donor and acceptor (here assigned the value of 2/3 as generally assumed in the literature [6]; and J is the spectral overlap integral between donor and acceptor, given by $J = \int F_\lambda^D \varepsilon_\lambda^A \lambda^4 d\lambda$, where F_λ is the normalized

donor fluorescence spectrum and ε_λ is the wavelength-dependent molar extinction coefficient ($M^{-1}\,cm^{-1}$) of the acceptor. For an isolated donor-acceptor pair, the FRET efficiency E is given by $E = [1 + (r + R_o)^6]^{-1}$.

RESULTS AND DISCUSSION

Molecular Design and Syntheses and Spectral Properties

The diheteroarylethene compounds selected as photoswitchable acceptors for FRET share the following properties: (i) The closed forms of the diheteroarylethenes have an absorption maximum in the range 540–570 nm. (ii) Both forms of the acceptor are thermally stable; and (iii) The compounds are available with a carboxylic acid moiety allowing their conjugation to amine or hydroxyl groups. The donor emission spectrum of the QD overlaps well the absorption of the closed but not the open forms of the diheteroarylethene DHE acceptor.

The computed critical transfer distance R_o for the closed forms of DHE was 45 Å. The corresponding values for the open form was 11 Å. The distance r_{DA} between the center of the QD and the central atom of the acceptor was estimated (from data supplied by the manufacturers and the crystal structure of streptavidin) as 26–56 Å; the range accounts for stereochemically feasible variations in the position of the 4 biotin binding sites of streptavidin. The computed FRET efficiency E of 0.96–0.23 reflects this uncertainty in r_{DA}. The efficiency E corresponding to the open form, was very low, i.e. 0.01–0.00. Both limits of E (open and closed forms) will be even more extreme in the case of multiple acceptors ($n > 1$) bound to a given QD.

Photochromic Conversion Analysed by Quantum Dot Fluorescence

I. Cyclic Modulation of Donor Fluorescence by Spectroscopy (Fig. 2)

The fluorescence signal corresponding to the QD suspension was monitored during irradiation with UV light (365 nm). The emission displayed an exponential increase, reaching a plateau after 30 min of irradiation. Subsequently, compound **4** was added and a further period (30 sec) of irradiation with 365 nm resulted in a decrease (34% quenching) in QD emission. Irradiation with visible light restored the emission signal to its initial value. Figure 2 depicts 28 transitions (alternating exposures to UV and visible light), demonstrating the reversibility and stability of the modulation.

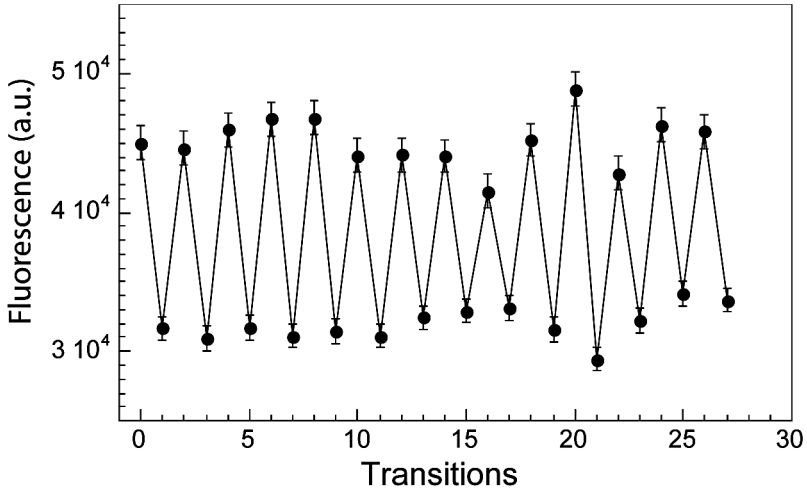

FIGURE 2 Reversibility of QD modulation by solution spectroscopy. Cycles of UV and visible irradiation. Qdot-565-streptavidin (4 nM) QDs were pre-irradiated with UV light for 40 min., after which the emission intensity was constant. Addition of 2.8 nM biotin-DHE and further irradiation with UV light for 30 s resulted in a decrease of the emission intensity, which reverted after exposure to visible light for 60 s.

II. Cyclic Modulation of Donor Fluorescence by Imaging

Streptavidin-conjugated magnetic microspheres were bound to polylysine-coated cover slips (μ-slides). The microspheres were then saturated with bis-biotin, washed, and exposed to a Qdot-565-streptavidin suspension. The μ-slides were thereby covered with sparsely distributed 2.6 μm magnetic beads, each of which was coated with QDs bridged to the carrier microspheres via bis-biotin, and by individual and clustered QDs distributed randomly over the surface. After washing, the slides were exposed to saturating levels of photochromic compound **4**.

The upper panel of Figure 3 displays a region including two microspheres. The QD emission was particularly intense on the surface of the microspheres due to their high local concentration, but was also observed for the individual and clustered QDs. An initial irradiation with UV light resulted in an increase of QD emission (representing a QD "curing" process), as was also observed in the spectroscopy measurements. After this phase, which we denote as pre-irradiation, each period of UV irradiation resulted in a systematic decrease in QD emission, which was restored to its initial value by subsequent exposure to

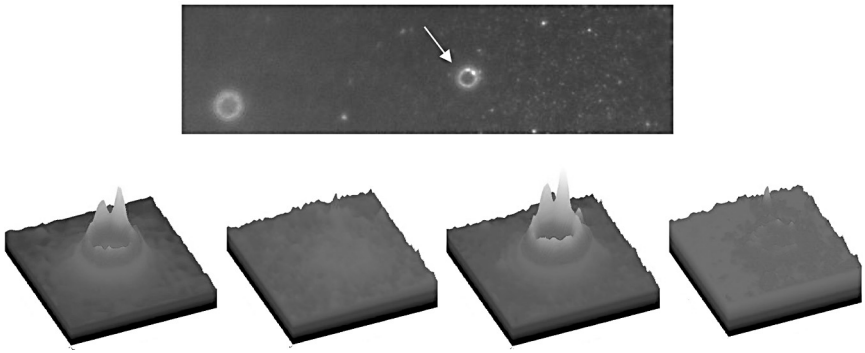

FIGURE 3 Imaging QD emission modulated by pcFRET. Polylysine cover slits were bound to streptavidin magnetic microspheres, washed with 50 mM phosphate-buffered saline (PBS), incubated for 10 min with 2.6 μM bis-biotin, washed, and incubated for 10 min with Qdot-565-streptavidin in PBS, and washed and incubated for 10 min with 2.5 μM biotin-DHE in PBS. *Upper panel*: The surface showed fluorescence shells (QDs) on the microspheres and also single and aggregated QDs bound directly on the surface. After a pre-irradiation period with UV and visible light the QDs displayed a reproducible decrease of emission upon irradiation with UV light (as a result of pcFRET) and an increase of emission when irradiated with visible light. *Lower panel*: Surface plots of a selected region containing a microsphere loaded with QDs (see arrow upper panel); from left to right: initial (after pre-irradiation phase), after UV, after visible, after UV exposure.

visible light. A series of images was acquired during UV and visible irradiation, from which we determined the kinetics of conversion under the given experimental conditions. The kinetic curves obtained for the conversion from the open to closed form as well as for the cycloreversion reaction were reproducible and repeatable, as monitored for the initial and final states by imaging with low-level 450 nm excitation.

Figure 3 features top and surface views corresponding to two cycles of conversion by UV and visible light of a selected microsphere (panel a, arrow). The intensity was modulated dramatically by the photoconversion, showing an almost 100% decrease in emission intensity. Certain areas containing QD aggregates displayed different degrees of quenching, reflecting inherent heterogeneity in the binding state of individual QDs, and of the microenvironment experienced by the QDs and the microsphere carriers. High sensitivity CCD cameras such as the novel electron multiplying unit employed here permit the observation of individual QDs.

CONCLUSIONS

We have previously demonstrated that diheteroarylethenes can function as efficient and "programmable" acceptors in FRET [2–7], a phenomenon exploited in countless biological and non-biological applications for determining molecular conformation and proximity. The photoreversibility of the photochromic acceptor provides unique advantages for performing repetitive and quantitative FRET determinations under the variable and unknown relative donor/acceptor stoichiometry generally prevailing in the microscopy of biological specimens. The cyclical determinations permit the evaluation of continuously evolving systems such as living cells during extended period of time.

In this study, we extended the pcFRET methodology to quantum dot donors and applied it for the first time in an imaging context. The only other report of photochromic switching of QDs [9] was of measurements in solution and employed a spiropyran/merocyanine photochromic system, the inherent properties of which are far inferior to those of the diheteroarylethenes; the diarylethenes with perfluorocyclopentenes have the capacity of undergoing $\geq 10^4$ cycles without sign of fatigue. The number of cycles of FRET activation-deactivation that can be achieved by cyclic irradiation with UV and visible light is of central importance in that this parameter determines the feasibility of carrying out continuous determinations in dynamic systems, e.g. with living cells. However, the diheteroarylethenes featured in this work have relatively low photoreversion quantum yields [4]. It would be advantageous to employ derivatives improved in this characteristic, as well as those offering forward photoconversion by blue light or by multiphoton excitation with pulsed lasers, thereby avoiding the potentially phototoxic effects of UV irradiation.

The primary motivation of this research has been the development of probes for biomolecules and cellular structures that can be tracked in living systems and manipulated so as to generate specific signals suitable for quantitative imaging with high spatial and temporal resolution. However "QD-pcFRET" may also constitute a valuable technology for implementing high-density optical memory devices.

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