

Small Photostable Photoswitchable Quantum Dots as Nanotools for Live Cell Imaging

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ABSTRACT

We recently reported a novel surface architecture for the generation of biocompatible and stable *photoswitchable* quantum dots (psQDs). The system is based on Förster Resonance Energy Transfer (FRET) from a QD donor to diheteroarylethenes photochromic (PC) acceptors (pcFRET). The PC molecules are linked to an amphiphilic polymer that self-assembles, creating an interior lipophilic space, in which the hydrophobic PCs are included, and external facing carboxyl groups. psQDs retain the desirable properties of the original QDs; furthermore the brightness can be tailored by light. The modulation of emission monitored by steady-state and time-resolved fluorescence is 35-40%. The epidermal growth factor receptor (EGFR) is of far-reaching biomedical importance due to its overexpression and/or mutation in various types of cancer. Here, we demonstrate imaging of live cells exposed to psQDs targeted to the EGFR.

Keywords: quantum dots, FRET, photochromic, EGFR, imaging.

1 INTRODUCTION

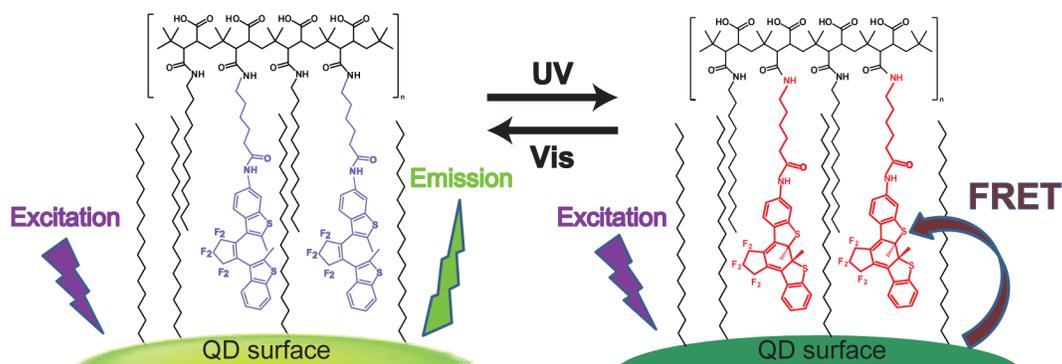
The imaging of living cells for the purpose of obtaining structural and dynamic information related to functional states is undergoing extensive development. The advancement involves improvements both in microscopy technology and in the engineering of new probes. Prominent in the latter category are functionalized quantum dots (QDs), which feature numerous superior properties: broad excitation, narrow emission, photostability, and high brightness [1]. Yet despite their virtues QDs can exhibit distinct limitations with respect to imaging *in vivo*: substantial size, nonspecific targeting, potential toxicity, and above all, limited means for cellular import. We present here a QD-based biosensor, which can be used *in vivo*, is smaller than traditional targeted QDs, can be targeted to specific cell surface receptors that then serve as carriers to the interior of the cell, and in addition is photoswitchable.

There are many examples in the literature of QDs functioning as Förster resonance energy transfer (FRET)

donors [2]. If certain spectroscopic criteria are met, the energy from an excited donor QD can be transferred to one or more proximal ground-state acceptors. Photochromic (PC) compounds are characterized by a reversible transformation between two different structural forms with distinct absorption spectra, when they are illuminated cyclically at appropriate wavelengths. If one of the absorption spectra of the PC overlaps the emission of a nearby fluorophore, the system exhibits switchable FRET, a process we have denoted previously as pcFRET [3, 4]. In this case the fluorescence emission of the QD donor can be modulated reversibly by manipulating the state of the PC acceptor with cycles of UV-visible irradiation. This allows greatly increased signal detection by rejection of background signals.

We built upon the concept put forth by Parak *et al* [5], to solubilize organic nanoparticles with amphiphilic comb-polymers, so as to create a water-soluble light modulatable QD [6]. The photoswitchable quantum dots (psQD) were formed by coating organic QDs with a comb-like photochromic amphiphilic polymer based on thermally stable diheteroarylethenes. This architecture permits the exploitation of the hydrophobic microenvironment, necessary for efficient PC conversion between the surface of the semiconductor QD and the external surface of the assembled nanoparticle. We placed the FRET acceptors in this compartment, thereby achieving two ends: provision of a chemical environment conducive to efficient photoswitching, and maintenance of close proximity to the QD donor. The fatigue due to the photobleaching of the donor or of the PC is minimal and thermal conversion of the acceptor is null at room temperature.

The epidermal growth factor receptor (EGFR), a member of the family of ErbB receptor tyrosine kinases, is of far-reaching biomedical importance due to its overexpression and/or mutation in various types of cancer [7]. The extracellular binding of Epidermal Growth Factor (EGF) activates various signaling cascades, for example the MAPK, Akt, and JKT pathways that regulate cell motility, differentiation, and division [8].



Scheme 1. Representation of photoswitching psQDs. Emission is modulated by modifying the photochromic FRET acceptors with UV or visible light.

The needs of extended real-time visualization, which has revealed important aspects of the binding, endocytosis and transport of the EGFR [9, 10] led to the development of QDs bearing EGF ligands. For these studies, biotinylated EGF was conjugated to commercial QD-streptavidin constructs. Although the semi-conductor crystal cores are small (~5 nm diameter) the addition of the passivation shell, polymers, streptavidin, and biotinylated EGF causes an increase in the final diameter to ~30 nm.

In this work we used the EGFR as a model system to demonstrate that biological molecules can be conjugated to psQDs, allowing for specific binding and cellular uptake. psQDs can be utilized in *in vivo* imaging with the aforementioned spectral advantage but now augmented with reversibly modulated fluorescence emission and lifetimes.

2 RESULTS AND DISCUSSION

2.1 psQD Preparation

The psQDs utilized in this work were optimized relative to those in a previous publication [6]. The starting QDs were commercially available CdSe/ZnS core-shell nanocrystals with octadecylamine ligands, (CZ520, NN-Labs, Fayetteville, AR). The previously reported photochromic polymers were formed by conjugating the aromatic amine of the diheteroarylethene photochrome to the backbone of polyisobutylene-alt-maleic anhydride (PMA; Sigma-531278, Mw ~6000) [6]. By modifying the PC with an aliphatic amine linker (see Methods) we increased the coupling yield from ~10 to ~50%. Due to the modification of the PC, the overlap between the absorption spectrum of the closed state of the acceptor and the emission spectrum of the QD increased slightly, leading to an increased Förster radius (R_0) of 4.1 nm, compared to 4.0 nm for the old QD-PC pair (version 1)[6]. We surmise that the new linker also places the PC up to 9 Å closer to the QD, such that the number of acceptors needed for achieving a given degree of quenching is reduced. Quantitative analyses of the data (see below) support this view. The

coating procedure for all QDs and purification steps followed that reported previously [6].

2.2 FRET Formalism

The FRET system operating in the psQDs is quite complex. The QD-donor has a heterogeneous shell of PC molecules with varying capabilities of functioning as FRET acceptors. We introduce in the analysis complexity in the form of two classes of closed photochromic acceptors (class 1, by definition, being more efficient) linked to a single QD donor [Eq. 1].

$$E_{i,j} = \frac{i\gamma_1 + j\gamma_2}{1 + i\gamma_1 + j\gamma_2}; q_{i,j} = 100E_{i,j} \quad (1)$$

$$\gamma_1 = \left(\frac{R_{o,1}}{r_{DA,1}}\right)^6; \gamma_2 = \left(\frac{R_{o,2}}{r_{DA,2}}\right)^6$$

where $E_{i,j}$ is the energy transfer efficiency in a psQD with i and j acceptors (class 1 and 2 respectively). The corresponding percentage quenching is $q_{i,j}$, $r_{DA,i}$ is the individual donor-acceptor (QD-PC group) separation and $R_{o,i}$ is the Förster transfer distance. The fractional photoconversion to the FRET-competent closed form is different for each class due to the different FRET efficiencies (greater FRET leads to a lower net photoconversion; see also Table 1 and reference [6] for an in-depth discussion of the system). The result is a heterogeneous mixture of responses to UV or visible irradiations within single psQDs. This heterogeneity was corroborated by single-particle spectroscopy (data not presented here).

Preparation:	psQD v1	psQD v2
QD quenching	41.4%	40.4%
% PC in polymer	5.9	3.2
# of acceptors (i, j)	31, 54	5, 36
$\gamma_1; \gamma_2$	0.48; 0.007	1.13; 0.03
$\alpha_1; \alpha_2$	0.04; 0.33	0.07; 0.35

Table 1. Parameters obtained from fitting photoconversion and QD quenching data comparing original (v1) and present (v2) preparations of psQDs. α_1, α_2 : fractional conversion to closed state of class 1 and 2 acceptors at photostationary state.

2.3 Conjugation of organic and biological molecules to psQDs

The presence of a large number of carboxyl groups on the exterior of the psQDs provide the means for conjugation to other molecules [11]. Experiments were initially realized with organic dye molecules, since they facilitated tracking. Using EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) chemistry we conjugated Texas Red cadaverine (Invitrogen T-2425). The addition of dyes to the exterior of the psQD did not affect the quenching of the QD. However, depending on the J overlap integral, pcFRET can exist between the PC and the QD as well as between the PC and the dye, as was observed with Texas Red.

The conjugations had to be optimized individually. At pH 8-9, excess EDC was added to compensate, although caution had to be exercised since excess EDC can cause crosslinking and/or irreversible aggregation.

Initial attempts at direct EGF conjugation resulted in a loss of activity of the molecule. This could be due to chemical reasons, modification of relevant lysine, or sterical reasons, inability of receptor pocket to envelop EGF. In an alternative approach, we conjugated streptavidin molecules to the psQD and utilized biotin-EGF (Invitrogen E-3477) for uptake. The extended linker allows for better presentation of the EGF to the EGFR. The robustness of streptavidin and its high affinity for biotin make it an effective system. For conjugation conditions used see METHODS.

2.4 Imaging EGF-conjugated psQDs in living cells

EGF conjugated psQDs were prepared by adding a 2-fold excess of biotin-EGF to streptavidin-psQD constructs and then diluting to the desired concentration. We utilized these constructs at 1 nM concentration to perform the internalization assays. Specific and significant binding and uptake was observed (Fig. 1A). Negative (psQDs and free EGF) and positive uptake controls (see [10]) were realized, and demonstrated neither unspecific binding and receptor uptake nor photoswitching.

Determinations of photocycling of the internalized psQDs were initiated. Due to limitations in the available wavelengths on the microscope we found it simpler to introduce quenched psQDs and then de-quench with green light (intense 546 nm emission of halide lamps). The response to irradiation was not homogeneous; intensity increased to different extents. Endosomes with high amounts of psQDs were more suitable for quantification due to their superior intensity and stability. Examples are shown in Fig. 1 BC with quantitation in panel D. The dequenching was 39.0 and 37.5% for points 1 and 2, in good agreement with the 40% obtained in bulk measurements.

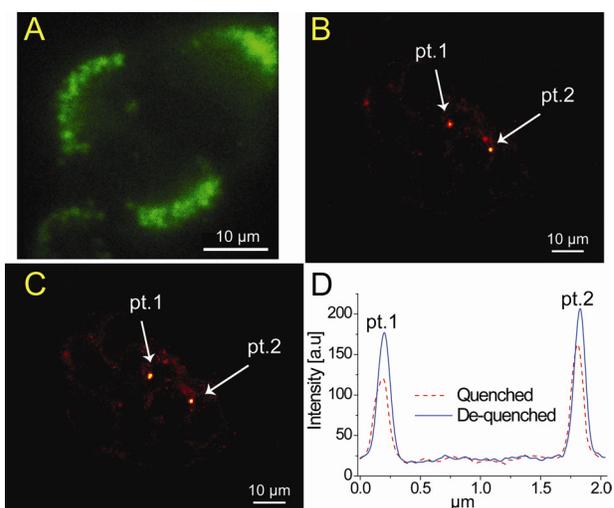


Figure 1. (A) Image of psQD-EGF conjugate uptake. (B) A431 cells with internalized quenched psQDs. (C) Same image as A after 30 seconds of 515-560 nm irradiation. (D) Intensity profile before and after irradiation.

3 METHODS

3.1 Synthesis of Photochromic polymer.

All reagents were from Sigma-Aldrich unless indicated otherwise.

1. *Synthesis of PC with aliphatic amine linker:* 6-amino-N-(3-(3,3,4,4,5,5-hexafluoro-2-(2-methylbenzo[b]thiophen-3-yl)cyclopent-1-enyl)-2-methylbenzo[b]thiophen-6-yl)hexanamide.

A solution of 3-(3,3,4,4,5,5-hexafluoro-2-(2-methylbenzo[b]thiophen-3-yl)cyclopent-1-enyl)-2-methylbenzo[b]thiophen-6-amine prepared in the lab (see [6]) was dissolved in dry CHCl_3 . It was then added to a flask containing 4 times the equivalents of freshly prepared tert-butyl 6-chloro-6-oxohexylcarbamate (from commercially available 6-(tert-butoxycarbonylamino)hexanoic acid, CAS: 6404-29-1). The reaction took place at 30 °C for 2 hours. The crude was extracted with CH_2Cl_2 and a green-brown oil was obtained. The BOC protective group was released from the aliphatic amine by addition of 20% fuming HCl in ethyl acetate for 10 minutes at room temperature. The reaction was quenched with NaOH and extracted with CH_2Cl_2 . The yield was 62%. The product was used to prepare amphiphilic photochromic polymer.

2. *Synthesis of photochromic amphiphilic polymer.*

PMA (60 mg) was added to a dry glass flask. The recently prepared PC (20 mg) was dissolved in anhydrous THF (2 ml) and added to the PMA in the flask. The added photochromic compound corresponded to a 2-fold excess over the anhydride monomers selected for conjugation with the photochromic moieties. The flask was sonicated for 2 minutes and left to react at 60 °C with stirring. The solvent was reduced to approximately half and a solution of dodecylamine in THF (48 mg) was added; the reaction

continued overnight. The preparation was dried and resuspended in anhydrous chloroform and purified from unreacted reagents using Sephadex LH-20 (GE Healthcare). The obtained polymer was vacuum dried (78.0 mg, yield 61%) and used for coating QDs.

3.2 Conjugation to psQDs

psQDs were stored in 50 mM Na-borate pH 9.0 buffer (SBB), and dyes or proteins were dissolved in SBB pH 7.3 (small amounts of DMF were added for solubility). EDC solutions were freshly prepared as 1 M solutions in SBB. The psQDs and conjugate target were mixed in the desired proportion. Successful conjugation required excess conjugate and high concentrations; sulfo-NHS (CAS: 106627-54-7) could be added to favor conjugation. EDC was added at ~2000 equivalents per conjugate. The reactions took place at room temperature for 10 minutes then quenched with 2-mercaptoethanol, after which the solution was dialyzed using Pierce Slide-A-lyzer Mini Dialysis units (10,000 MWCO) or purified through exclusion column once more.

3.3 Microscopy

A431 cells (expressing 2.3×10^6 EGFR molecules on their membrane) were labeled with 1 nM EGF-QD constructs and incubated for 30 minutes. psQDs were irradiated with 340 ± 10 nm light (1.3 mW/cm^2) for 45 seconds either before incubation or once uptake was completed to ensure that they were in the quenched form. Confocal laser scanning microscopy was performed with a Leica TCS SP5 using a 63×1.4 NA oil immersion objective (Fig. 1 B,C) or with a Gen-2 Programmable Array Microscope (PAM) (Fig. 1A) [12]. QDs were excited with a 405 nm diode laser, a wavelength at which photoreversal of the PC was minimal; the QD emission peak was at 545 nm. The psQDs were de-quenched by utilizing a FRAP mode with 514 or 561 nm lasers, or a Hg arc lamp with a 515-560 band pass filter.

4 CONCLUSIONS

We have demonstrated the use of psQDs to realize cellular imaging and the capability to switch the state of psQDs during the imaging. The ability to modify the psQDs directly to the exterior carboxyl groups opens a wide range of possibilities in targeting as well as functionalization. In the case of EGF direct conjugation will require additional steps but it remains a viable option for other molecules.

As was shown the improvement of psQDs is an ongoing project. To reach their full potential the heterogeneity of the psQDs must be resolved to utilize them as single particle probes and a shift towards longer wavelengths would facilitate cellular imaging.

The interweaving of nanotechnology and biology will continue for the foreseeable future and the creation of new

tools, such as psQDs, will provide a scaffold with which new applications and methodologies can be developed.

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