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BODIPY-Based Donor/Donor-Acceptor System: Towards Highly Efficient Long Wavelength-Excitable Near-IR Polymer Dots with Narrow and Strong Absorption Features

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Abstract

Bright long-wavelength-excitable (green and beyond) semiconducting polymer dots (LWE-Pdots) are highly desirable for *in vivo* fluorescent imaging, as well as applications of multiple-wavelength-excitation based *in vitro* bioassays. Conventional LWE-Pdots have been obtained previously by incorporating a small amount of near-infrared (NIR) emitter into the backbone of a polymer host to develop a binary donor-acceptor (D-A) system. They usually suffer from severe concentration quenching and a trade-off between fluorescence quantum yield (Φ_f) and absorption cross-section (σ), limiting the improvement in brightness. In this communication, we describe a ternary component (D_1/D_2 -A) strategy to achieve ultrabright, green laser (532 nm)-excitable Pdots with narrow-band NIR emission by introducing a BODIPY-based assistant polymer donor as D_1 . The novel use of BODIPY as predominant absorber in a Pdot allows strong, narrow-band absorption at 532 nm. The D_1/D_2 -A Pdots possess simultaneously improved Φ_f and σ compared to corresponding binary D_2 -A Pdots. Their Φ_f is as high as 40.2%, one of the most efficient NIR Pdots reported. The ternary D_1/D_2 -A Pdots show ultrahigh single-particle brightness, ~83 times brighter than Qdot 705 when excited by a 532 nm laser. Intravenously injected Pdots accumulate in implanted tumors in mice through the enhanced permeability and retention effect. Higher

Conflict of interest

S. R. Hingorani, is a consultant for Halozyne Therapeutics. J. Yu and D. T. Chiu have financial interest in Lamprogen, which has licensed the described technology from the University of Washington.

contrast *in vivo* tumor imaging was achieved using the ultrabright ternary Pdots versus the binary D-A Pdots. What's more, this D₁/D₂-A design strategy supplies us a promising way to pursue ultrabright Pdots with even longer absorption and emission.

Graphical Abstract

Achieving long-wavelength-excitable near-infrared semiconducting polymer dots with simultaneously high fluorescence quantum yield, strong absorption cross-section, narrow absorption-emission bandwidths, and large Stokes-shift by employing a BODIPY-based donor/

Keywords

Semiconducting polymer dots; BODIPY; Narrow-band absorption; Near-infrared; *In vivo* imaging

Over the past few years, semiconducting polymer dots (Pdts) have emerged as a new class of fluorescent probes for cellular imaging, sensors, *in vivo* imaging, and optical theranostics.^[1–13] Most reported Pdts with high fluorescence quantum yield (Φ_f) were based on blue light excitable polymer host, such as poly(9,9-dioctyl-2,7-fluorene) (PFO, 405 nm laser) and poly(fluorene-alt-benzothiadiazole) (PFBT, 488 nm laser).^[14–17] However, for *in vivo* imaging, long-wavelength-excitable (LWE) Pdts with preferred near-infrared (NIR) emission^[18–25] are desirable to increase the excitation and emission light penetration through tissue, to minimize the impact of tissue autofluorescence.^[26,27] On the other hand, simultaneous identification of numerous biological species by a single experiment^[28–30] are important to minimize the sample consumption and running times for cell research, disease diagnosis and treatment. LWE-Pdts with narrow absorption and emission bands are therefore expected to be highly useful in applications involving multi-wavelength-excitation

based *in vitro* assays such as cell barcoding,^[30] flow cytometry,^[31] and quantitative polymerase chain reaction.^[32]

Poly[(9,9-dioctyl-fluorene)-alt-(4,7-di-2-hexylthienyl-2,1,3-benzothiadiazole)] (PFDHTBT: $\lambda_{\text{abs, max}} \sim 520$ nm) is a classic green laser excitable polymer donor. The PFDHTBT-based Pdots show moderate Φ_f around 15%.^[33,34] Yet reported Pdots with $\lambda_{\text{abs, max}}$ beyond 520 nm always show low Φ_f (0.1% ~1.7%),^[35–38] mainly because of the severe fluorescence concentration quenching effect in solid-like Pdot state. LWE Pdots with low Φ_f are promising for *in vivo* photoacoustic imaging^[39–41] or photothermal therapy^[42,43] due to effectively non-radiative decay, but not for high contrast fluorescence imaging. To alleviate aggregation-induced self-quenching, polymers have been designed with twisted molecular structures and/or bulky steric groups, and the resulting far-red^[44] and NIR Pdots^[34] have achieved higher Φ_f values (20% and 33%, respectively). However, comparing to PFDHTBT donor, the improved Φ_f were obtained at the cost of a lower 532 nm absorption cross-section (σ), due to reduced absorbing ability (poorer molecular planarity and/or lower density of absorbing moieties) and blue-shifted absorption peak in cyan region (~ 495 nm). Because probe brightness is proportional to the product of Φ_f and σ , the trade-off between them limited the improvement in brightness. Moreover, all existing LWE Pdots exhibit broad absorption bands. Therefore, it is a great challenge to develop LWE Pdots with simultaneously high Φ_f and strong absorption as *in vivo* fluorescence contrast agent, and additional narrow-band absorption and emission for the specific applications in multiplexed *in vitro* assays.

In this work, we describe the first BODIPY-based NIR Pdot with simultaneously high Φ_f and strong σ at 532 nm, narrow absorption and emission bandwidths, and large Stokes-shift features. Higher contrast *in vivo* tumor imaging was achieved using the ultrabright ternary D₁/D₂-A Pdots versus the binary D-A Pdots.

As shown in Figure 1, a small amount of BDP720 acceptor (3 mol%) was incorporated into a PFDHTBT polymer backbone, and the resulting binary D-A Pdot (PFDHTBT-BDP720, 0.005 g L⁻¹ solution) showed a moderate Φ_f of 17.7% and an absorbance of 0.11 (Table 1). The primary polymer donor (PFGBDP, D₁) was prepared by grafting green BODIPY (GBDP) dye to the side-chain of PFO. PFGBDP showed a Φ_f of 85% in THF solution, but was almost non-emissive in the Pdot state (Φ_f of 0.3%) due to formation of H-aggregation dimers (Figure S1). In addition, the overlap between PFGBDP emission and NIR dye absorption spectra was poor, causing inefficient Forster energy transfer (FRET). Thus, PFGBDP could not be used independently as the host material of the NIR acceptor for a D-A type Pdot. However, considering its strong absorbance at 532 nm (Figure 2a), we used PFGBDP as the primary donor (D₁) in a ternary Pdot, blending it with an optimized 20 wt% PFDHTBT-BDP720 polymer (SI, section 11). As shown in Table 1, the resulting ternary Pdot had an absorbance of 0.23 (0.005 g L⁻¹ solution) and a high Φ_f of 40.2%, 2.1- and 2.3-fold greater than the binary Pdot, respectively, resulting in a 4.7-fold increase in brightness.

The high Φ_f of the ternary PFGBDP/PFDHTBT-BDP720 Pdots can be attributed to two factors: a) efficient cascade energy transfer from GBDP through PFDHTBT to BDP720

emitter; and b) restricted self-quenching of PFDHTBT and BDP720 due to their low concentration in the Pdots.

The energy-transfer efficiency (Φ_{ET}) from the assistant GBDP donor to PFDHTBT-BDP720 was estimated to be 91% (SI, section 12). In addition to good spectral overlap between PFGBDP/PFDHTBT (D_1/D_2) and PFDHTBT/BDP720 (D_2/A) (Figure 2b), two other factors facilitated efficient cascade energy transfer. First was the large amount of PFDHTBT (~20 wt%) embedded in the Pdots. GBDP H-dimers can form in Pdots due to parallel plane-to-plane stacking, resulting in quenched fluorescence (Figure 2a). In the absence of PFDHTBT, excited GBDP monomer and dimer (S_1') rapidly fall into the dimer's lower energy level (S_1''), which is dipole-dipole forbidden for radiative emission (Figure 2c, green arrows). To compete with the non-emissive GBDP dimer species, a large amount of PFDHTBT was introduced to maximize capture of energy from excited GBDP monomers and dimers *via* FRET. In addition, the high PFDHTBT content resulted in a small average distance between GBDP and PFDHTBT; in this case, even a GBDP H-dimer in the S_1'' state could transfer its energy to PFDHTBT by short-range energy transfer ($r < 1$ nm) *via* electron exchange coupling or orbital overlap between donor and acceptor electronic densities.^[45,46] The second factor that facilitated efficient energy transfer was direct incorporation of BDP720 emitter into the PFDHTBT backbone. Since the BDP720 content in the Pdot was low, energy transfer in the ternary Pdot predominantly occurred *via* a cascade from GBDP to PFDHTBT to BDP720 emitter, incorporation BDP720 into the PFDHTBT backbone facilitated FRET between these two species. Furthermore, covalent conjugation of the donor and acceptor also enabled through-bond energy transfer.^[17]

In addition to efficient cascade energy transfer, the high Φ_f of the ternary Pdot system can be attributed to suppressed self-quenching of PFDHTBT and BDP720. Pdots composed only of PFDHTBT or PFDHTBT-BDP720 showed low Φ_f (11.2% and 17.7%, respectively) (Table S1). After dispersing 20 wt% PFDHTBT or PFDHTBT-BDP720 into a PFO Pdot host (with no absorption at 532 nm), the Φ_f at 532 nm excitation improved to 23.1% and 44.0%, respectively.

One important criterion for evaluating fluorescent probes is single molecule/particle brightness. The theoretical brightness is proportional to the product of σ and Φ_f . The single-particle photophysical properties of the ternary Pdots, binary PFDHTBT-BDP720 Pdots, and water soluble PEGylated NIR quantum dot (Qdot 705) are shown in Table 2. Based on theoretical estimation, the ternary Pdots were expected to have single-particle brightness ~4.3-fold greater than the binary Pdots due to the improved σ and Φ_f , and to be ~100-fold brighter than Qdot 705 when excited at 532 nm. Because single-particle brightness is sensitive to particle size, we also calculated the expected brightness on a per volume basis for the three NIR nanoparticles. The ternary and binary Pdots were predicted to be 13.4-fold and 2.7-fold brighter, respectively, than Qdot 705 per volume when normalized to size of water soluble Qdot 705.

Single-particle images of the three NIR nanoparticles were acquired by using a wide-field total internal reflection microscope (Figure 3). Based on these images, the mean-averaged single-particle brightness of the ternary Pdot, binary Pdot, and PEGylated Qdot 705 were

calculated to be 3.89×10^5 , 9.78×10^4 , and 4.69×10^3 photons/frame, respectively. The ternary Pdot was 4.0 fold brighter than the binary Pdot and 83-fold brighter than Qdot 705, close to the theoretical values. Moreover, unlike the broad absorption from ultraviolet to visible-red of Qdot 705, the ternary Pdots possessed a narrow absorption band with a full width at half maximum (FWHM) of 50 nm, roughly half that of the binary Pdot (99 nm). The ternary Pdots also exhibited a narrower emission bandwidth (46 nm FWHM) than Qdot 705 (72 nm). The narrow absorption and emission bandwidths as well as the large Stokes-shift (193 nm) make these ternary Pdots ideal for multiplexed bioassay applications.

The *in vivo* imaging performance of the Pdots was examined in tumor-bearing nude mice injected with Pdots *via* the tail vein. Whole-body fluorescence images were collected at various time intervals following injection (Figure 4a). Immediately after injection, nanoparticles are typically sequestered by the mononuclear phagocyte system (MPS), resulting in low whole-body fluorescence. However, PEGylated Pdots such as used in this study are masked from the host's immune system by the PEG groups, reducing MPS uptake and increasing the blood circulation time.^[47–49] Mice injected with PEGylated Pdots have previously shown higher fluorescence whole-body images than mice injected with non-PEGylated nanoparticles.^[33]

During the 48 hours following tail vein injection, both binary and ternary Pdots accumulated in the tumor due to their long blood circulation time and due to the enhanced permeability and retention (EPR) effect.^[50,51] However, consistent with the greater brightness of the ternary Pdots versus the binary Pdots, the whole-body fluorescence brightness and ratio of tumor to surrounding tissue fluorescence were greater when using the ternary Pdots (Figure 4b). After surgically opening the abdominal skin, strong fluorescence signals were detected in the lymph nodes and tumor, and the margins of the lymph nodes and tumor were clearly discernible (Figure 4c). Therefore, the ternary Pdots appear suitable for use as a contrast agent for image-guided surgery during tumor and sentinel lymph node resection. The biodistribution of the Pdots was evaluated 48 hours after tail vein injection. Representative fluorescence images of organs including the liver, lung, spleen, kidney, and heart are shown in Figure 4d. The injected Pdots were found mainly in the liver, lymph nodes, and tumor. The Pdot distribution was analyzed quantitatively using fluorescence signals from organs in Pdot-injected mice (n=3) (Figure 4d). The prominent retention of Pdots at tumor sites makes Pdots promising for use as a contrast agent for tumor imaging or as a drug carrier for cancer treatment.

In conclusion, by employing a BODIPY-based donor/donor-acceptor strategy, a green laser-excitable NIR Pdot with simultaneously improved Φ_f and σ was developed. The ternary ultrabright LWE Pdot was roughly five-fold brighter than the corresponding binary Pdot, and allowed higher contrast *in vivo* tumor imaging in mice. More importantly, we believe this D₁/D₂-A design strategy can be extended to develop bright Pdots having even longer wavelength absorption and emission spectra for use in bioimaging and multiplexed biological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.
Polymer structures and design mechanism of ultrabright 532 nm-excited NIR Pdot

Figure 2.

(a) Absolute absorption (Abs; solid line) and fluorescence (FL; dash-dot line) spectra of 0.005 g L^{-1} PFGBDP Pdots (black), PFDHTBT-BDP720 Pdots (blue), and blended Pdots (red); (b) Normalized absorption and photoluminescence (PL) spectra of PFGBDP and PFDHTBT Pdots, and BDP720 dyes in Pdot state; (c) Energy levels of GBDP monomer, GBDP H-dimer, PFDHTBT, and BDP720 in Pdot state, and the cascade energy transfer between them.

Figure 3. Single particle imaging and brightness distribution of Qdot 705, the binary Pdot, and the ternary Pdot.

Figure 4.

In vivo imaging of tumor-bearing nude mice using PEGylated Pdots. a) Fluorescence images of mice injected with binary Pdots or ternary Pdots. b) Ratios of fluorescence signal of tumor to background in the initial 48 hours after Pdot injection. c) Bright-field and fluorescence images of the same mouse after opening of the peritoneum 24 hours post Pdot injection. d) Quantitative fluorescence analysis of the Pdots in liver (Li), lung (Lu), spleen (Sp), kidney (K), heart (H), tumor (T), lymph nodes (LN) (n=3). Inset shows the overlap image of fluorescence image and bright-field image of liver, lung, spleen, kidney, heart, tumor, lymph nodes, and intestine (In) from binary Pdot and ternary Pdot injected mice.

Table 1.

Photophysical properties and size of Pdots.

Pdot	$\lambda_{\text{abs, max}}$ (nm)	$\lambda_{\text{PL, max}}$ (nm)	A ^a	Φ_{f} (%)	Size ^c (nm)	ζ Potential (mV)
PFGBDP	528	548	0.28/0.27	0.3	31.4	−29.4
Binary	520	724	0.11/0.10	17.7 ^b	32.6	−50.4
Ternary	528	721	0.23/0.22	40.2 ^b	31.0	−43.7

[a] Absorbance of 0.005 g L^{−1} Pdots at the absorption peak and at 532 nm.

[b] Φ_{f} was acquired from 650 to 850 nm.

[c] Number-averaged diameter measured by dynamic light scattering.

Table 2.
Photophysical properties of three NIR nanoparticles (Qdot 705, binary Pdots, ternary Pdots).

NIR NPs	$\lambda_{\text{abs,max}}$ (nm)	$\lambda_{\text{PL,max}}$ (nm)	FWHM (nm)	ϵ_{532} nm (M ⁻¹ cm ⁻¹)	σ_{532} nm (cm ²)	$\phi_{\text{t,NIR}}$ (%)	Brightness ($\Phi_{\text{t}} \times \sigma$, cm ²)	Size (nm)	Brightness per volume ($\Phi_{\text{t}} \times \sigma / V$, cm ⁻¹)
Qdot 705 ^a	< 300	707	72	2.1×10^6	8.0×10^{-15}	79.8 ^b	6.4×10^{-15}	15.8 ^b	3091.1
Binary Pdots	520	724	48	2.2×10^8	8.3×10^{-13}	17.7	1.5×10^{-13}	32.6	8303.3
Ternary Pdots	528	721	46	4.2×10^8	1.6×10^{-12}	40.2	6.5×10^{-13}	31.0	41600.5

^aQdot 705 data were obtained from Thermal Fisher Scientific.

^bMeasured under the same conditions as Pdots.