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# Deep-red polymer dots with bright two-photon fluorescence and high biocompatibility for *in vivo* mouse brain imaging

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ABSTRACT

With high contrast and deep penetration, two-photon fluorescence (2PF) imaging has become one of the most promising *in vivo* fluorescence imaging techniques. To obtain good imaging contrast, fluorescent nanoprobes with good 2PF properties are highly needed. In this work, bright 2PF polymer dots (P dots) were applied for *in vivo* mouse brain imaging. Deep-red emissive P dots with PFBT as the donor and PFDBT5 as the acceptor were synthesized and used as a contrast agent. P dots were further encapsulated by poly(styrene-co-maleic anhydride) (PSMA) and grafted with poly(ethylene glycol) (PEG). The P dots-PEG exhibit large two-photon absorption (2PA) cross-sections (δ≥8500 g), good water dispersibility, and high biocompatibility. P dots-PEG was further utilized first time for *in vivo* vascular imaging of mouse ear and brain, under 690–900 nm femtosecond (fs) laser excitation. Due to the large 2PA cross-section and deep-red emission, a large imaging depth (~720 μm) was achieved.

## 1. Introduction

Fluorescence nanoprobes have played an important role in bioimaging, due to their convenience to usage, as well as excellent sensitivity [1,2]. To improve the spatial resolution of bioimaging, many efforts have been made to develop various types of fluorescence microscopy [3–5]. Among these techniques, two-photon fluorescence (2PF) imaging had become one of the most prominent, for their deeper penetration depth, weaker tissue autofluorescence, less photodamage and less photobleaching [6–9]. Apart from this, to improve the collection efficiency of fluorescence signals, emissions in deep-red/near-infrared (NIR) region are advantageous because the scattering caused by tissue would be smaller for longer wavelengths emissions [10,11]. So nanoprobes with both of their excitations and emissions in the deep-red/NIR region are eagerly needed. These nanoprobes include upconversion nanoparticles, organic dyes and many other nanoparticles, while most of them have poor 2PF intensity or poor biocompatibility [12,13].

Semiconducting Polymer dots (P dots) are new generation of promising fluorescence nanoprobes for their super fluorescence brightness, good photostability and excellent biocompatibility [14–17]. Thence, considerable efforts have been made to synthesize various types of P dots with distinct emissions [18,19]. Meanwhile, P dots with good 2PF properties (high 2PA cross-section and high quantum yield) are still in great need [20,21]. Recently, researchers had synthesized some P dots with good 2PF properties, while there was no systematic work on *in vivo* brain vascular imaging yet [22,23].

In this paper, bright 2PF P dots with donor-acceptor (D-A) structure were utilized for *in vivo* mouse brain imaging. PFBT with good visible light harvesting were used as the donor and PFDBT5 with deep-red emission was used as the acceptor [24,25]. Then they were further encapsulated with poly(styrene-*co*-maleic anhydride) (PSMA) to form polymer matrix. The matrix structure endowed P dots with two important features: deep-red emission and high quantum yield. P dots were further conjugated with long-chain poly(ethylene glycol) (PEG) molecules, to avoid the capture by the reticuloendothelial/degradation

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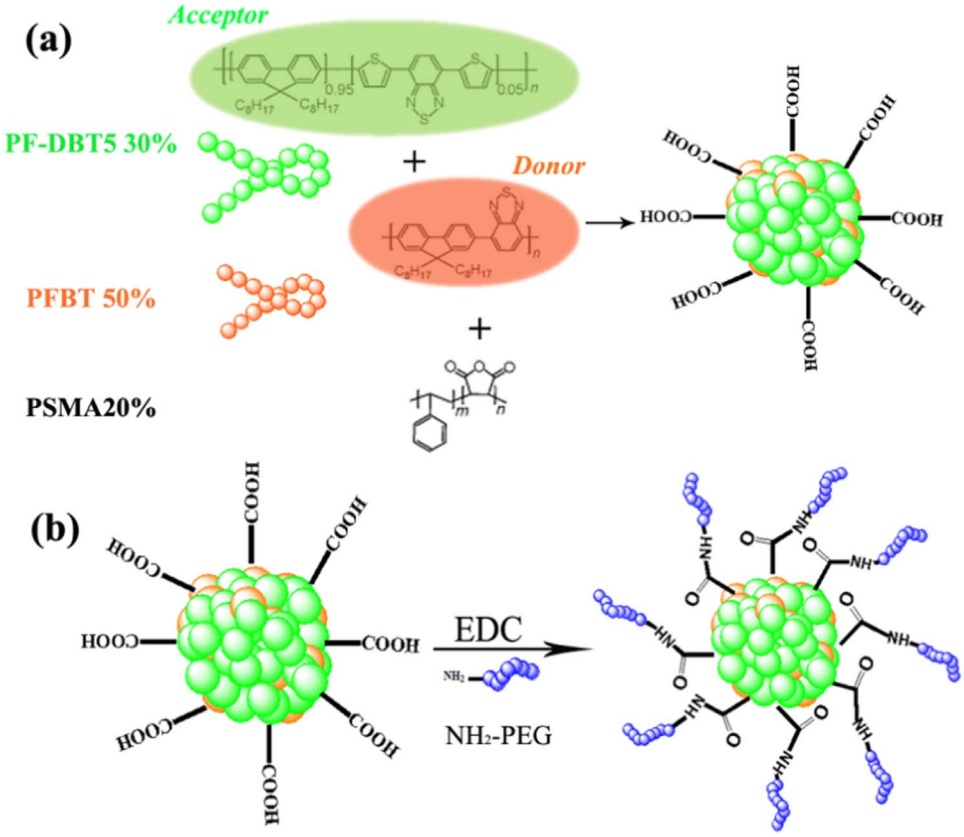


**Fig. 1.** Synthesis and functionalization routine of P dots. (a) Synthesis process of P dots, (b) Encapsulating P dots with $NH_2$-PEG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

system and to increase the circulation time [26,27]. After that, chemical stable and biocompatible P dots were obtained. The 2PA cross-sections of P dots were measured under 720–960 nm fs laser excitation, and they were found to have the largest value at 810 nm fs laser excitation. P dots were then used as fluorescence probes for 2PF imaging and *in vivo* 2PF imaging of mouse ear and brain angiography was realized under a commercial 2PF imaging system (720–960 nm). A large penetration depth of 720 μm was obtained under the excitation of 810 nm fs laser excitation, mainly due to the large 2PA cross-section and the deep-red emission of P dots.

## 2. Experimental section

### 2.1. Materials and methods

Functionalized P dots in aqueous solution were prepared by using a modified nanoprecipitation method according to literature [25], as shown in Fig. 1a. The prepared P dots consist of donor-acceptor polymer blends, in which the visible-light harvesting polymer PFBT is the donor and the efficient deep-red emitting polymer PFDBT5 is the acceptor. The blending ratio of P dots is 0.6 (PF-DBT5 to PFBT in weight), and at this ratio P dots exhibits a broad visible absorption band as well as an efficient deep-red emission (650 nm) with a high quantum yield of 57% [25]. Surface bioconjugation was then performed by utilizing the EDC-catalyzed reaction between carboxyl P dots and the amine-containing $NH_2$-PEG, as shown in Fig. 1b. The synthesized P dots would possess bright deep-red fluorescence and good biocompatibility.

The absorption spectra of P dots were measured by a Shimadzu UV2550 UV–vis–NIR scanning spectrophotometer. Morphologies of P dots were captured by a JEOL JEM-1200EX transmission electron microscope (TEM) operated at 160 kV. TEM samples were prepared by casting 10 μL of P dots aqueous solution on copper grids and dried at room temperature. The 1PF ($\lambda_{excitation}$=450 nm) and 2PF spectra of P dots ($\lambda_{excitation}$=810 nm fs laser excitation) were measured by a home-built system (in Fig. S2) and were collected by a spectrometer (PG2000, Ideaoptics Instruments).

### 2.2. Animals' preparation

All the animal experiments were performed strictly in compliance with the requirements and guidelines of the Institutional Ethical Committee of Animal Experimentation of Zhejiang University. The ICR mice (~18 g, female) were obtained from the Laboratory Animal Center of Zhejiang University (Hangzhou, China). The mice were housed in cages at 24 °C with a 12 h light/dark cycle and were fed with standard laboratory water and chow.

### 2.3. Histology

24 h and 50 days after the injection of P dots (in 300 μL 1× PBS, 1600 ppm), experimental mice (injected with P dots), as well as control mice (without P dots injection), were sacrificed. Their major organs (heart, liver, lung, kidney, brain and spleen) were removed for histological analysis. Tissue samples were harvested and fixed in 4% paraformaldehyde overnight at 4 °C. Then the samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E). The histological sections were imaged under an inverted optical microscope (with a 20×objective lens).

### 2.4. 2PA cross-section measurements

Two-photon action cross-section ($\eta\delta$) is a parameter describing how bright a fluorophore is under two-photon excitation [24,28]. It is