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Amplification of near-infrared fluorescence in semiconducting polymer nanoprobe for grasping the behaviors of systemically administered endothelial cells in ischemia treatment

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ABSTRACT

To date, there have been few studies on using fluorescent cell trackers for non-invasively monitoring the *in vivo* fate of systemically administered cells. This is because only a relatively small number of cells can reach the disease site post systemic infusion, and thus achieving ideal *in vivo* cell tracking requires that the fluorescent cell trackers should hold combined merits of ultrahigh near-infrared (NIR) fluorescence, negligible interference on cell behavior and function, excellent retention within cells, as well as accurate long-term cell tracking ability. To address this challenge, we herein developed a highly NIR fluorescent nanoprobe (SPN) based on semiconducting π-conjugated polymers (SPs), by synthesis of a NIR SP-emitter, employment of fluorescence resonance energy transfer (FRET) strategy, and optimization of different FRET donor SPs. Due to the 53.7-fold intra-particle amplification of NIR fluorescence, the SPN could track as few as 2000 endothelial cells (ECs) upon intra-arterial injection into critical limb ischemia (CLI)-bearing mice, showing much higher sensitivity in ECs tracking compared with the most popular commercial cell trackers. What's more, the SPN could provide precise information on the behaviors of systemically injected ECs in CLI treatment including the *in vivo* fate and regenerative contribution of ECs for at least 21 days.

1. Introduction

Ischemia, a restriction in blood supply to tissues, often causes tissue damage or dysfunction [1]. Among many an ischemic disease such as myocardial infarction, stroke, critical limb ischemia (CLI), and so on [2–4], CLI is an advanced stage of peripheral artery disease that is highly associated with diabetes mellitus. The patients suffering diabetes are faced with a heavy risk of major amputation caused by CLI [5,6]. Thereby, a great amount of research and clinical efforts have focused on cell-based therapy strategies (*e.g.*, stem cells, endothelial progenitor cells, or endothelial cells) for CLI treatment, showing the significant outcome in improving limb perfusion [7–9]. With the flourishing progress of cell therapy, effective exogenous cell trackers that can report the *in vivo* fate of administrated cells in an accurate and long-term manner are in urgent pursuit. Although a variety of imaging contrast agents have been developed as cell trackers, rather limited studies can clearly prove that whether their agents still precisely label the original cells *in vivo* over an exceedingly long period of time and how the cells dedicate to the disease therapy by virtue of the agent labeling [10].

To achieve ideal long-term cell tracking, a sensitive imaging modality with high resolution in combination with a biostable and biocompatible contrast agent are required. Compared with versatile imaging techniques, fluorescence imaging holds the advantages

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of excellent sensitivity, high resolution at cellular level, maneuverable instruments, and good safety [11]. In particular, bioimaging in the near-infrared (NIR) window (650–900 nm) offers the merits of low interferential autofluorescence and high tissue permeability [12–14]. In comparison to traditional fluorescent materials, semiconducting π-conjugated polymers (SPs) have been well accepted as a class of advanced fluorescent materials with high extinction coefficient, bright emission, large photobleaching threshold, and good biocompatibility [15–19]. Recently, SP-based nanoparticles (SPNs) have received considerable attention as they integrate both the advantages of SPs and nanotechnology [20–42]. As compared to inorganic quantum dots (QDs), SPNs have been reported to show higher fluorescence by tens of times, better biological stability, and much lower cytotoxicity [43–45], which make them very promising as next generation of fluorescent nanodots.

In clinical cell therapy, systemic infusion including intravenous (i.v.) and intra-arterial (i.a.) injections is the most common method for administration of cells [46]. Therefore, understanding the behaviors of systemically administered cells in disease treatment becomes increasingly important and urgent. Until now, however, there have been few published studies on using fluorescent cell trackers for non-invasively monitoring the *in vivo* fate of systemically administered cells. This is because only a relatively small number of cells can reach the disease site post systemic infusion, and thus achieving ideal *in vivo* cell tracking requires that the fluorescent cell trackers should hold combined merits of ultrahigh NIR fluorescence, superb biological and photophysical stabilities, negligible interference on cell behavior and function, excellent retention within cells, as well as precise long-term cell tracking ability [47–50]. This longstanding challenge motivated us to explore advanced fluorescent cell trackers to meet all the aforementioned requirements.

In this contribution, we reported the development of a highly NIR fluorescent SPN for close monitoring of *in vivo* behaviors of systemically administered human umbilical vein endothelial cells (ECs) in the treatment of CLI. A NIR SP-emitter, PFDBD10 (Fig. 1A), was synthesized and used as the fluorescence resonance energy transfer (FRET) acceptor. Two visible-light-harvesting SPs, PFBD and PFBT (Fig. 1A), were utilized as the FRET donor candidates. Using a facile nanoengineering method, the donor and acceptor SPs were co-formulated into one nanoparticle to realize FRET, achieving tremendously amplified NIR fluorescence. Importantly, we demonstrated that PFBD could serve as a better FRET donor than PFBT in terms of higher absorptivity at 488 nm, larger enhancement of acceptor emission, and higher photoluminescence (PL) quantum yield (QY) of SPNs in water. After surface functionalization using an EC proteoglycan-binding peptide, the PFBD/PFDBD10-PBP-SPNs were applied to trace systemically administered ECs in a CLI-bearing mouse model. By virtue of the ultrahigh NIR brightness, PFBD/PFDBD10-PBP-SPNs could not only accurately and dynamically track the ECs after i.a. or i.v. injection for as long as 3 weeks, but also show much higher sensitivity in tracing of ECs *in vivo* than the most popular commercial cell trackers, Qtracker® 655 and PKH26. Besides, the PFBD/PFDBD10-PBP-SPNs labeling was able to reveal how the systemically administered ECs devote to CLI therapy *in vivo*, offering important information on EC therapy of ischemia.

2. Materials and methods

2.1. Synthesis of PFDBD10

A Schlenk tube was charged with compound **1** (192.6 mg, 0.3 mmol), compound **2** (131.5 mg, 0.24 mmol), compound **3** (26.5 mg, 60.0 μmol), as well as $Pd(PPh_3)_4$ (4.6 mg, 4.0 μmol) in toluene (8.0 mL) before it was sealed with a rubber septum. The Schlenk tube was degassed with three freeze-pump-thaw cycles to remove air. After the mixture was heated to 85 °C, an aqueous Et_4NOH solution (20 wt%, 2.0 mL) was added to initiate the reaction. The mixture was kept at 85 °C and stirred for 20 h, followed by precipitation from methanol (100 mL). The precipitate was collected by filtration and dried, and then dissolved in chloroform (200 mL). The solution was then washed with water for 3 times, and dried over $MgSO_4$. After solvent removal, the residue was poured into methanol (100 mL) to give polymer as fibres. The polymer was further purified by Soxhlet extraction in acetone for 24 h to remove the fraction with small molecular weight. The precipitation cycles in toluene/methanol were then repeated twice to afford the final polymer (142.6 mg, 63%) as bright red fibres. ^1H NMR (600 MHz, $CDCl_3$, ppm) δ: 8.17 (br), 7.84 (br), 7.78–7.67 (br), 7.52 (br), 7.37 (br), 2.12 (br), 1.25–1.14 (br), 0.82 (br).

2.2. Preparation of SPNs

Predetermined amounts of donor SP (PFBD or PFBT), and/or PFDBD10 (fixed at 0.3 mg) as well as maleimide-bearing lipid-PEG (fixed at 3 mg) were dissolved in 1 mL of tetrahydrofuran (THF), which was dropped into 9 mL of water under sonication using a microtip probe sonicator at 12 W output. The mixture was then sonicated for another 60 s. After evaporation of THF, the SPN suspensions were filtered through a 0.45 μm syringe driven filter. The amounts of donor SP/PFDBD10 encapsulated into the lipid-PEG matrix were determined from the absorption spectra with reference to a calibration curve of donor SP and PFDBD10 in THF. Generally, the encapsulation efficiency of SP defined as the ratio of the amount of SP encapsulated into the SPNs to the total amount of SP in the feed mixture was >90%. To conjugate proteoglycan-binding peptides (PBPs) on the SPNs, 0.2 μmol peptides were added into the SPN suspension, which was allowed to react for 12 h. The free PBPs without conjugation on SPNs were subsequently removed by ultrafiltration.

2.3. Cell culture

Human umbilical vein endothelial cells (ECs) were maintained in EGM-2 (endothelial cell growth medium-2) medium (Lonza, USA) in a 37 °C incubator under a humidified 95% air and 5% CO_2 atmosphere. The culture medium was changed twice a week. The ECs of passage 3 were used for all the experiments in this work.

2.4. In vitro tracking of ECs

ECs (2×10^6) were seeded in a 6-well plate and cultured at 37 °C. After removal of the medium and washing with 1 × PBS buffer, the ECs were exposed to 1 nM PFBD/PFDBD10-PBP-SPNs for 4 h at 37 °C. After that, the ECs were washed twice with 1 × PBS buffer, digested by tripsin-EDTA, and then subcultured for 1, 5 and 10 days, respectively. The ECs subcultured for different time points were imaged by confocal laser scanning microscopy (CLSM; Leica TSC SP8, Germany) with excitation at 488 nm and signal collection above 560 nm (pinhole: 65.5 μm). The fluorescence intensities of SPN-labeled ECs were then assessed by flow cytometry (Becton Dickinson, San Jose, CA, USA) measurements. Ten thousand events were counted for each sample to plot the histogram (excitation at 488 nm; 680/20 nm bandpass filter). The *in vitro* ECs tracking study using commercial Qtracker® 655 as a control was also performed following the same procedures.

2.5. Capillary tube formation in vitro

PFBD/PFDBD10-PBP-SPN-labeled or untreated ECs were