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ROS Self-Scavenging Polythiophene Materials for Cell Imaging

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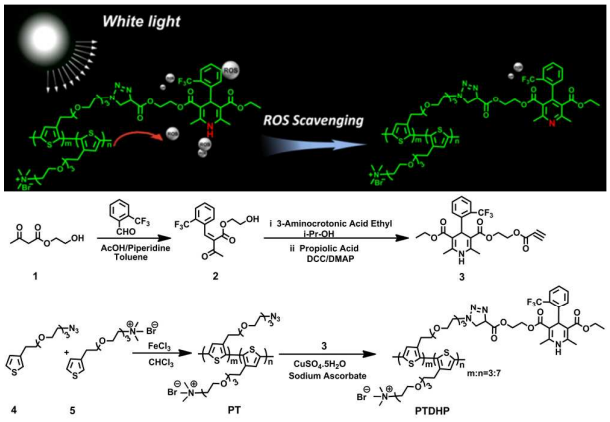
A conjugated polymer (PTDHP) was synthesized by modifying the side chain of cationic polythiophene (PT) with dihydropyridine (DHP) group via click reaction. PTDHP has unique ROS self-scavenging ability through oxidation of DHP into pyridine-structure upon light irradiation. Thus, PTDHP achieves cell imaging with good photo-stability and low photo-cytotoxicity.

Sensitively fluorescent materials have been abstracted more attention not only in fundamental biology but also in clinical diagnosis.[1-3] Among these fluorescent materials, the two most commonly used ones are organic dyes and quantum dots.[1-5] However, rapid photobleaching of organic dyes[6] and heavy-metal related cytotoxicity of quantum dots[7] make it still necessary to develop new fluorescent materials. Conjugated polymers (CPs) with π-electron delocalized backbones exhibit unique light-harvesting ability and high optical signal amplification effect, which have been extensively studied for highly sensitive chemical and biological sensing.[8-15] In addition, CPs possess high fluorescence brightness and excellent photostability, thus they have been widely used in live cell imaging.[16-28] However, obstacles still remain because CPs can sensitize surrounding oxygen to generate reactive oxygen species (ROS) exposing to light, which not only bleaches material fluorescence, but also is harmful to organism and unfavourable for cell imaging.[29-33] Thus new strategies for increasing photo-stability and reducing photo-toxicity of CPs are required.

Dihydropyridine (DHP) derivatives are a most studied class of calcium channel blockers to limit the calcium influx by binding to dihydropyridine receptor on cell membrane. Besides their clinic use in treatment of hypertension, they also exhibit antioxidative effect.[34-37] DHP derivatives can scavenge ROS by oxidation of the dihydropyridine ring into pyridine-structure.[38,39] In this study, we synthesized a novel ROS self-scavenging CPs (PTDHP) by modifying the side chain of cationic polythiophene (PT) with DHP group via click reaction. The PT was used due to its low cytotoxicity without light irradiation.[40,41] The PTDHP could realize cell imaging via binding to the cell membrane through electrostatic and hydrophobic interactions, also it is capable of reducing photo-toxicity as DHP group can delete ROS generated by polythiophene upon light irradiation for imaging through oxidation into the pyridine-structure.



Scheme 1. Schematic illustration of ROS self-scavenging of PTDHP and its synthetic route.

The photo-cytotoxicity of conjugated polymers results from their sensitization of oxygen molecules through excited energy transfer to readily produce reactive oxygen species (ROS) for rapidly killing neighboring living cells/organism upon light irradiation. The in-situ generated ROS could also damage the polymer structure to bleach their fluorescence, which leads to low photo-stability. As shown in **Scheme 1**, cationic conjugated polymer (PTDHP) contains polythiophene backbone and dihydropyridine (DHP) side chain. Upon light irradiation, the polythiophene part could sensitize oxygen molecules to generate ROS, while the ROS could be consumed by DHP group through oxidation of dihydropyridine into pyridine-structure. Thus the unique ROS self-scavenging ability of PTDHP offers it

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good photo-stability and low photo-cytotoxicity for cell imaging.

The synthesis of PTDHP is outlined in **Scheme 1**. The compound **2** was obtained by reacting 2-(trifluoromethyl)benzaldehyde with compound **1** in the presence of acetic acid and piperidine with a 50% yield. Then, compound **3** was prepared through cyclization of compound **2** with 3-amino-2-butenoic acid ethyl ester followed by modification with alkynyl group by reacting with propynoic acid in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) with a yield of 53%. The NMR and the high resolution mass spectra of compound 2 and 3 are shown in Figure **S2**. **PT** was obtained by oxidative polymerization of monomers **4** and **5** with $FeCl_3$ in chloroform followed by dialysis in water via a membrane with a molecular weight cutoff of 3500 g/mol with a yield of 26%. Finally, **PTDHP** was prepared by linking compound **3** to **PT** side chain via click reaction followed by dialysis in water and precipitation in ethyl acetate to give an orange solid. Based on ^{1}H-NMR spectroscopy of PTDHP, the content of DHP is calculated to be about 25%. The weight-average molecular weight (M_w) of **PT** was measured to be 73,600, while that of **PTDHP** was 94,400 based on GPC analysis using polystyrene as the standard with DMF as the eluent.

As shown in **Figure 1a**, PT displays a maximum absorption at 425 nm in water, while that of PTDHP exhibits a blue shift (at 390 nm) compared to PT due to the modification with DHP moiety. The maximum emissions of PT and PTDHP are both around 570 nm with fluorescence quantum yields of 5% in water with quinine sulfate as the standard. Since both PT and PTDHP possess hydrophilic cationic side chains and hydrophobic skeletons, they are expected to form aggregates in water. Their aggregations were further investigated by dynamic light scattering (DLS). **Figure 1b** and **Figure 1c** show that after modification with DHP moiety, PTDHP exhibits a larger aggregate size (mean diameter: 29.1 nm) in comparison with that of PT (mean diameter: 13.8 nm) in water. In order to verify that DHP group could scavenge ROS by itself, ^{1}H-NMR spectroscopy of DHP were measured before and after addition of H_2O_2. As shown in **Figure 1d**, the proton in dihydropyridine-ring disappears after reacting with H_2O_2, which reveals the formation of pyridine-structure upon oxidation of dihydropyridine-ring. Thus the DHP are active enough to react with ROS, and it is expected that DHP group could protect the backbone of PTDHP from ROS to reduce photo-bleaching.

To investigate the photo-stability of ROS self-scavenging PTDHP, the PT and PTDHP with identical concentration were exposed to white light at a dose of 6 mW·cm^{-2} for 14 minutes, and the fluorescence intensity was recorded every minute as shown in **Figure 2a**. It is obvious that the fluorescence intensity of PTDHP decreases more slowly than that of PT, which demonstrates better photo-stability of PTDHP and 70% emission remains upon white light irradiation for 14 minutes. Considering that polymer will sensitize the surrounding oxygen molecules to generate ROS resulting in cell damage upon exposure to white light, we speculate that PTDHP will exhibit lower photo-cytotoxicity because of the ROS scavenging by DHP group in comparison to PT. To confirm this hypothesis, the cytotoxicities of PT and PTDHP in the dark and under light

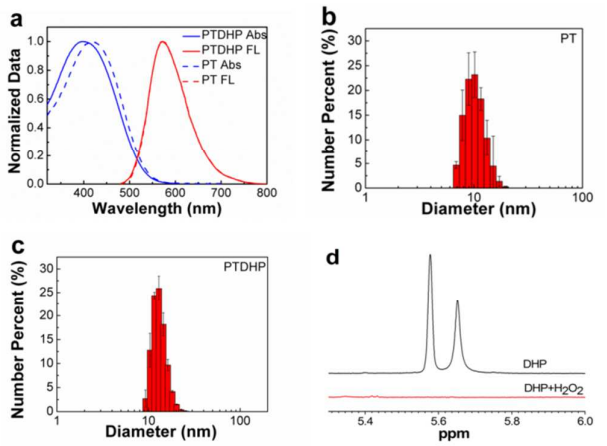

Figure 1. (a) Normalized absorption and fluorescent emission spectra of PT and PTDHP in water. The excitation wavelengths of PT and PTDHP are 460 nm and 450 nm, respectively. (b-c) Size distribution histograms of PT and PTDHP measured with DLS. (d) ^{1}H NMR spectra of DHP before and after addition of H_2O_2.

were both studied. In the dark, we investigated the intrinsic cytotoxicity of PTDHP and PT towards rat aortic endothelial cells. **Figure 2b** indicates that both PT and PTDHP possess lower cytotoxicity in the concentration range of 0 ~ 32 μM, and 20 μM is chosen as the concentration for further experiments under light. Photo-toxicity experiments of PT and PTDHP were performed upon exposure to 1 mW·cm^{-2} white light for 15 min. As shown in **Figure 2c**, it is evidently that PT shows a severer photo-toxicity for rat aortic endothelial cells compared to PTDHP as we expected. The results indicate that PTDHP possesses favorable anti-oxidative effect and better biocompatibility for fluorescence imaging.

To investegate the ultimate location of PTDHP and PT in rat aortic endothelial cells, cell imaging experiments were conducted by using confocal laser scaning microscopy (CLSM), followed by colocalization with organelle-specific staining dyes and line series analysis. As shown in **Figure 2d**, after incubation of PTDHP with living cells for 9 h, the fluorescent images for PTDHP and DiD (membrane dye) merged well, relatively few changes in the emission intensity profiles and a high pearson's coeffcient (0.82) were obtained, while non-merged images and low pearson's coeffcient (0.59) for PTDHP and LysoTracker were observed (**Figure 2e**). These results confirm that the polymer is mainly bound to the cell membrane, and only few were uptaken into lysosome, and the similar phenomena were found for PT (**Figure S1**). As shown in **Figure 2f**, well merged fluorescnet images and a relatively high pearson's coeffcient for DHP and LysoTracker (0.90) demonstrate a perfect colocalization, even though, a actually low pearson's coeffcient for DHP and DiD (0.12) is observed (**Figure S1**). The results

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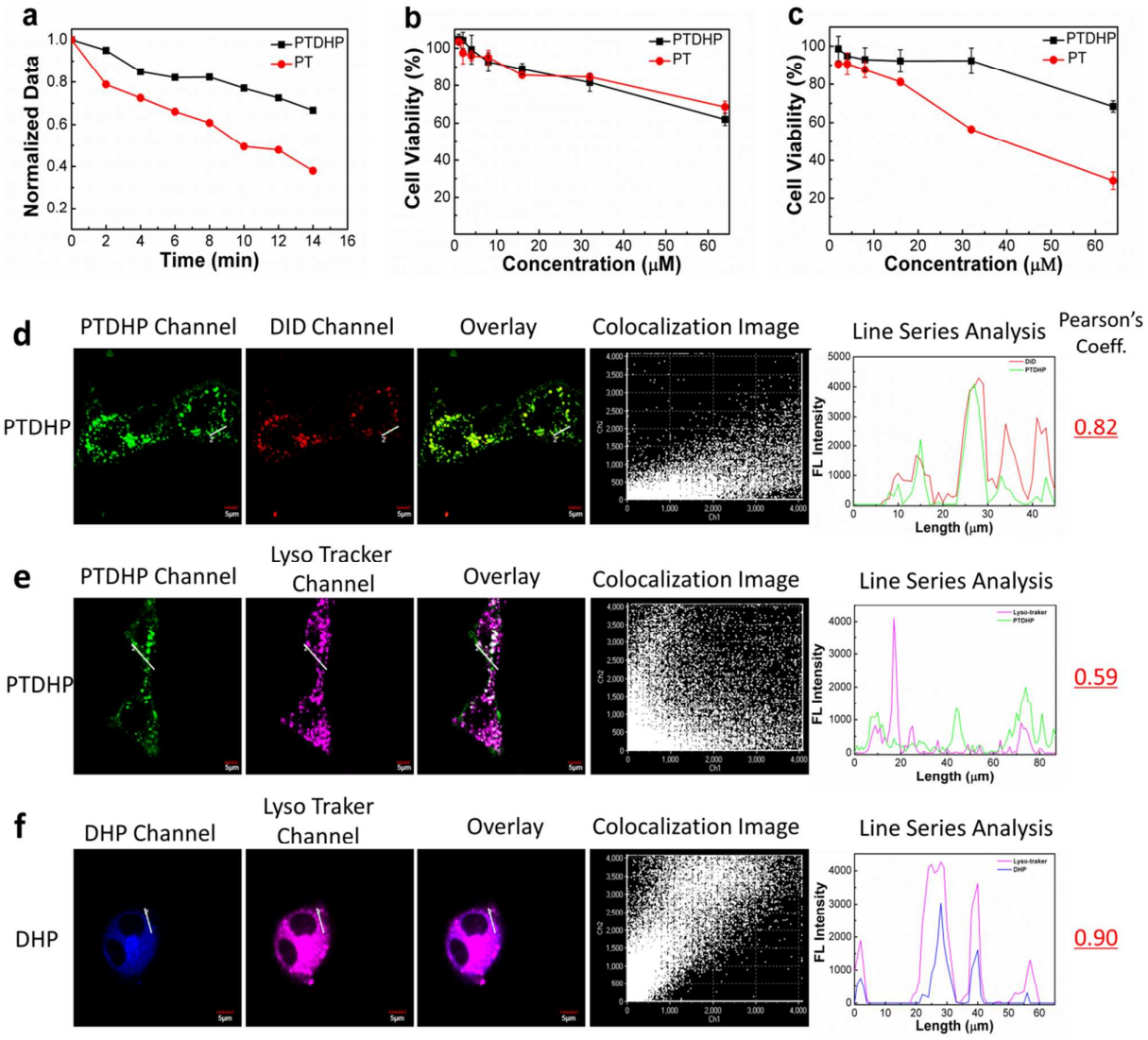

Figure 2. (a) Photo-stability of PT and PTDHP upon exposure to 6 mW·cm^{-2} white light for 14 min. [PT or PTDHP] = 3.0 × 10^{-5} M in repeat units (RUs). (b) Cell viability of rat aortic endothelial cells incubated with PTDHP and PT for 24 h in the dark, and (c) upon exposure to 1 mW·cm^{-2} white light for 15 min. [PT or PTDHP] = 2.0 ~ 64.0 × 10^{-6} M. (d) CLSM images of rat aortic cells, colocalization of PTDHP with DiD and line series analysis within ROI after incubation with PTDHP for 9 h and DiD for 0.5 h at 37 °C. [PTDHP] = 2.0 × 10^{-5} M in RUs. [DiD] = 5.0 × 10^{-6} M. PTDHP was highligted in green, DiD was highlighted in red. Fluorescence images of rat aortic endothelial cells, colocalization of PTDHP with LysoTtracker probe and line series analysis within ROI after incubation with PTDHP (e) and DHP (f) for 9 h, followed by treatment with LysoTracker for 1 h at 37 °C. [PTDHP] = 2.0 × 10^{-5} M in RUs. [LysoTracker] = 5.0 × 10^{-7} M. [DHP] = 5.0 × 10^{-6} M. PTDHP was highlighted in green, LysoTracker was highlighted in magenta, DHP was highlighted in blue. Colocalization, line series analysis and pearson's correlation coefficient were evaluated by OlympusFluoview.

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mean that DHP was located in lysosome of rat aortic endothelial cells after incubation for 9 h, which is absolutely distinct from that of PTDHP. The different distributions of PTDHP and DHP indicate that the moddification of DHP to PT was benifical for the combination and long retention of DHP to binding sites on the cell membrane.

Conclusions

In conclusion, we have designed and synthesized a cationic polythiophene (PTDHP) with dihydropyridine (DHP) group on the side chain via click reaction. PTDHP has unique ROS self-scavenging ability for the DHP group can delete ROS generated from polythiophene upon light irradiation through oxidation into pyridine-structure. Thus the DHP group can protect the backbone of PTDHP from ROS to reduce photo-bleaching and enhance photo-stability of PTDHP. PTDHP also exhibit lower photo-cytotoxicity because of the ROS self-scavenging. This work opens a new avenue to design multifunctional polymers for enhanced cell imaging with good photo-stability and low photo-cytotoxicity.

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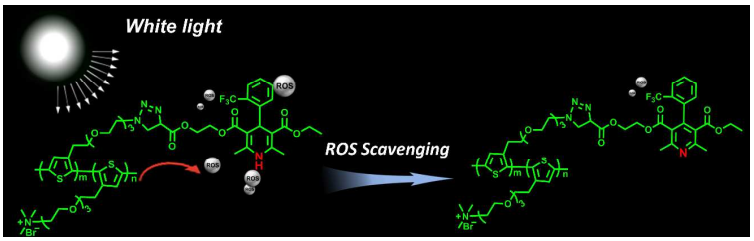

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