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Synthesis of a water soluble red fluorescent dye and its application to living cells imaging

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ABSTRACT

A water soluble red fluorescent dye (**TD-mPEG$_{750}$**) has been prepared by treatment of 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene)malononitrile with 4-(diethylamino)-2-mPEG benzaldehyde (mPEG-OH, average MW = 750). **TD-mPEG$_{750}$** exhibits red emission at λ_{em} = 664 nm in water, a small fluorescence quantum yield (ϕ_f = 0.01) and a large Stoke's shift ($\Delta\lambda$ = 145 nm) are obtained. Using HeLa cells as prototype, the application of **TD-mPEG$_{750}$** to living cells imaging has been investigated. It is found that **TD-mPEG$_{750}$** can be clearly expressed in mitochondria with high contrast in HeLa cells imaging.

1. Introduction

The use of water-soluble fluorophore dyes has become a significant area of research in biomedical diagnosis and biological image [1–8]. Fluorescent dyes with red emission ($\lambda_{em} \geq 650$ nm) are highly desired in biological imaging due to their particular advantages such as large penetration depth, less light scattering and minimized tissue auto-fluorescence background [9–12]. Common fluorophore dyes such as fluorescein, rhodamine, and quinine sulfate exhibit short emission wavelength ($\lambda_{em} \leq 600$ nm) [13–15], which limited their application in biological imaging. Recently, a number of noted red fluorophore dyes including BODIPY [16–20], cyanine dyes [21–24] and others [25–27] have been developed, but one main problem is encountered with them: small Stoke's shift ($\Delta\lambda \leq 70$ nm).

Development of water-soluble red fluorophore dyes with large Stoke's shift is essential for biological applications [28–31]. Advances in fluorescent dyes with large Stoke's shift not only reduce the self-quenching resulted from the molecular self-absorption due to the overlap between absorption and emission spectral of dyes but also can be used in multiplex monitor since monitoring multiple physiological parameters require the loading of several distinct fluorescent probes in the intracellular and extracellular environments [32], in which fluorophores that are excitable at the same fixed wavelength with well-separated emissions are required.

Dicyanoisophorone derivatives have currently attracted considerable attention because of red emission and large Stoke's shift [33–36]. Herein, a water-soluble fluorescent dye **TD-mPEG$_{750}$** based on dicyanoisophorone system (Scheme 1) has been designed and synthesized. The fluorophore **TD** is easy prepared and shows good photo-stability, the introduction of methyoxypolyethylene glycol (mPEG$_{750}$) to **TD** is to improve the solubility of **TD** in water and to decrease cytotoxicity. Poly(ethylene glycol) (PEG) have been extensively studied for their potential biomedical applications as scaffolds in tissue engineering [37,38] and as drug delivery systems [39,40] due to their biocompatibility, nontoxicity, and biodegradability [41,42]. In this paper, the properties of **TD-mPEG$_{750}$** and its application to cells imaging are examined, some merits are obtained, they include:

- Facile preparation.
- Good solubility in water.

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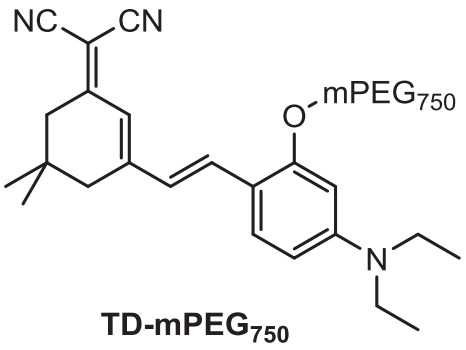

Scheme 1. Chemical structure of **TD-mPEG$_{750}$**.

➢ Deep red fluorescence.
➢ Large stoke's shift.

2. Experimental

2.1. General

^{1}H and ^{13}C NMR spectra are recorded at 400 and 100 MHz, respectively, with TMS as an internal reference. MS spectra are recorded with MALDI-MS spectrometer. UV absorption spectra and fluorescence spectra are measured with an absorption spectrophotometer (Hitachi U-3010) and a fluorescence spectrophotometer (F-2500), respectively. All experiments are carried out with commercially available reagents and solvents, and used without further purification, unless otherwise noted.

2.2. Experiment for cell culture and fluorescence images

For the fluorescence imaging in live cells, HeLa cells are cultured in culture media Dulbecco's modified Eagle's medium (DMEM/F12 1:1 (HyClone) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin) at 37 °C under a humidified atmosphere containing 5% CO_2 for 24 h. The cells were seeded on a Ø 35 mm glass-bottomed dish (NEST) for live-cell imaging by confocal laser scanning microscopy (CLSM). The HeLa cells were treated with 1 μM of **TD-mPEG$_{750}$** in 2 mL of serum free medium for 2 h and imaged by CLSM without removing the molecule in the cell medium. Confocal fluorescence imaging was performed with Nikon multiphoton microscopy (A1R MP) with a 60 × oil-immersion objective lens (NA = 1.40) and living cell work station. The cellular images were taken under a CLSM by using the excitation channel at 561 nm.

2.3. Experiment for toxicity test

Toxicity test of HeLa cells incubated with **TD-mPEG$_{750}$** is carried out as follows: (a) HeLa cells were incubated with 1 μM of **TD-mPEG$_{750}$** for 2 h, after washed up 3 times with phosphate buffered saline (PBS), 1 mL of fresh PBS was added. (b) To the incubated HeLa cells in PBS was added propidium iodide (PI) probe, after incubation for 10 min, the HeLa cells with **TD-mPEG$_{750}$** and PI probe were washed up with PBS for three times, 500 μL of fresh PBS was then added. (c) The sample was observed by Nikon A1R confocal fluorescence microscope with excitation wavelength of 561 nm, and the range of collected fluorescence is 570–620 nm. (d) The number of dead cells (red) and the whole number of cells were counted from the obtained images. Around 200 cells were counted, and the ratio of living cells (viability, %) was calculated. The viability of the cells without incubation of **TD-mPEG$_{750}$** was also checked by Plunder under the same experimental condition. The viability (%) of stained cells is calculated by relation to that of unstained cells in which the viability of unstained cells is set to 100%.

2.4. Synthesis of *TD-mPEG$_{750}$*

The synthetic route for **TD-mPEG$_{750}$** is outlined in Scheme 2, and the detailed procedures are as follows: (a) To a solution of isophorone (3.8 g, 27.6 mmol) and malononitrile (1.82 g, 27.6 mmol) in dry ethanol (150 mL) was added piperidine (23 mg, 0.276 mmol). The solution was stirred at 60 °C till starting material disappeared (detected by TLC plate). After cooling to room temperature, the solution was slowly poured into water (200 mL) and the precipitated solid was filtered. Recrystallization from heptane afforded 2-(3,5,5-trimethylcyclohex-2-enylidene) malononitrile as a brown solid. Yield: 4.5 g (90%). M.p. 73–75 °C. ^{1}H NMR ($CDCl_3$): δ (ppm) 6.60 (s, 1H), 2.53 (s, 2H), 2.14 (s, 2H), 2.01 (s, 3H), 1.32 (s, 6H). ^{13}C NMR ($CDCl_3$): δ (ppm) 170.3, 161, 120.2, 113.1, 76.4, 45.6, 42.3, 32.4, 27.5, 25.1. (b) To the solution of methoxypolyethylene glycol ($mPEG_{750}$-OH) (7.5 g, 10 mmol) in $CHCl_3$ (30 mL) was added thionyl chloride (2.5 g, 21 mmol) and pyridine (1.6 g, 20 mmol), the solution was refluxed till no starting material was detected (TLC detection). After cooled down to room temperature, the solution was poured into water (100 mL) and extracted with $CHCl_3$ (30 mL × 3). The combined organic solution was dried over Na_2SO_4, after evaporation of the solvent, the product **mPEG$_{750}$-Cl** (oil, 6.5 g, 85% yield) was obtained for next step without purification. (c) To a solution of 4-(diethylamino)-2-hydroxybenzaldehyde (0.96 g, 5 mmol) in DMF (10 ml) was added **mPEG$_{750}$-Cl** (3.8 g, 5 mmol), K_2CO_3 (0.7 g, 5 mmol) and KI (0.08 g, 0.5 mmol). The mixture solution was heated at 100 °C till no starting material was detected (TLC detection). After evaporation of DMF under pressure, 20 ml of H_2O was added to the mixture. The mixture was extracted with DCM (20 mL × 3), the combined organic solution was dried over Na_2SO_4, after evaporation of the solvent, **DA-mPEG$_{750}$** (oil, 2.26 g, 50% yield) was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 10.09 (s, CHO), 7.65 (d, J = 8.8 Hz, Ar–H), 6.85–6.81 (m, Ar–H), 6.22 (d, J = 8.8 Hz, Ar–H), 3.81–3.45 (m large, PEG backbone), 3.37–3.32 (q, N–$\underline{CH_2}CH_3$), 3.31 (s, –O–CH_3), 1.14 (t, N–$CH_2\underline{CH_3}$). (d) Under argon, 2-(3,5,5-trimethylcyclohex-2-enylidene) malononitrile (0.46 g, 2.5 mmol) and **DA-mPEG$_{750}$** (2.26 g, 2.5 mmol) were dissolved in dry acetonitrile (10 mL). Piperidine (2.1 mg, 0.025 mmol) was added and the solution was stirred at 40 °C till starting material disappeared (detected by TLC plate). After evaporation of acetonitrile under pressure, 20 ml of H_2O was added to the mixture. The mixture was extracted with DCM (20 mL × 3), the combined organic solution was dried over Na_2SO_4, after evaporation of DCM, the target compound **TD-mPEG$_{750}$** (oil, 2.7 g, 50% yield) was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.34 (d, J = 8.8 Hz, Ar–H), 7.32 (d, J = 16.0 Hz, CH=CH), 7.02 (d, J = 5.2 Hz, Ar–H), 6.86–6.81 (m, Ar–H), 6.69 (s, Ar–H), 6.24 (d, J = 8.8 Hz, Ar–H), 6.06 (d, J = 2.0 Hz, CH=C), 3.61–3.56 (m large, PEG backbone), 3.37–3.32 (q, $\underline{CH_2}CH_3$), (3.31 (s, –O–CH_3), 2.51 (s, CO–CH_2–), 2.16 (s, –CH_2–), 1.32 (s, CH_3–C–CH_3), 1.14 (t, N–$CH_2\underline{CH_3}$). ^{13}C NMR (100 MHz, $CDCl_3$: δ = 158.3, 155.6, 151.4, 136.7, 134.3, 131.8, 129.0, 128.5, 124.1, 115.3, 113.3, 112.0, 78.3, 78.8, 70.51 (br PEG), 65.4, 57.4, 41.2, 40.3, 37.6, 31.7, 28.1, 14.4. IR (KBr) ν (cm^{-1}) = 3122, 1640, 1589, 1470, 1455, 1240, 1112 (br).

3. Results and discussion

3.1. Synthesis of *TD-mPEG$_{750}$*

TD-mPEG$_{750}$ is obtained from isophorone, malononitrile and the corresponding aromatic aldehydes by a two-step condensation