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Thiophene-based fluorescent probes with low cytotoxicity and high photostability for lysosomes in living cells

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ABSTRACT

Background: Selective imaging of lysosomes by fluorescence microscopy using specific fluorescent probes allows the study of biological processes and it is potentially useful also for diagnosis. Lysosomes are involved in numerous physiological processes, such as bone and tissue remodeling, plasma membrane repair, and cholesterol homeostasis, along with cell death and cell signaling. Despite the great number of dyes available today on the market, the search for new fluorescent dyes easily up-taken by cells, biocompatible and bearing bright and long-lasting fluorescence is still a priority.
Methods: Two thiophene-based fluorescent dyes, **TC1** and **TC2**, were synthetized as lysosome-specific probes.
Results: The new dyes showed high selectivity for fluorescent staining and imaging of lysosomes and disclosed high photostability, low toxicity and pH insensitivity in the range 2–10.
Conclusions: The **TC** dyes exhibited high co-localization coefficients (>95%) and moderate quantum yields. They showed high biocompatibility and long-term retention, important features for biological applications.
General significance: The results of the present work disclose a new class of organic dyes with potential wide applications as specific and efficient lysosome probes in the study of various biological processes.

1. Introduction

Lysosomes (**Lyso**), which are roughly spherical bodies enclosed by a single membrane, are an important class of cellular organelles that receive and degrade macromolecules from the secretory, endocytic, autophagic, and phagocytic membrane-trafficking pathways [1]. Defects in lysosome function lead to the development of diseases with often-severe consequences for individuals. Since the discovery of lysosomes by Christian de Duve over 50 years ago [1–5], research into endocytic and lysosomal biology has allowed the development of tools to understand further the role of lysosomes in cells.

Fluorescence microscopy, which allows non-invasive imaging of organelles in live cells, is one of the most commonly used imaging technologies in cell biology. Selective imaging of **Lyso** by fluorescence microscopy using specific fluorescent probes would enable further studies in the aforementioned biological processes, and potentially also for diagnosis [6–9]. A fluorescent marker must have the capability to be internalized into the cells with long-term retention as well as long-lasting fluorescence. More importantly, the probe should be biologically inert and non-toxic. Owing to these requirements and despite the great number of dyes available today on the market, the search for new fluorescent dyes easily up-taken by cells, biocompatible and bearing bright and long-lasting fluorescence is still a priority. Several fluorescent dyes with visible wavelengths for **Lyso**, such as LysoTracker®, LysoSensor™, have been produced and are commercially available [10]. Nevertheless, many of them suffer from various practical limitations, such as a poor light fastness. This being so, a significant demand remains for lysosomal selective probes with high bio-and photostability, low cytotoxicity and long-wavelength emission peaks for a

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deeper tissue penetration. Fluorophores based on thiophene core have been widely used as organic dyes for efficient fluorescent cell staining [11], labeling of proteins and DNA [12]. This widespread use is due to the high photostability of the fluorophore skeleton, low cytotoxicity, high color-tunable emission, high quantum efficiency and large Stokes shifts. Noteworthy, these dyes have a robust aromatic backbone, which can undergo a variety of functionalizations, including bio-derivatization [12–15].

Herein, we report the synthesis and investigation of two biocompatible thiophene-based fluorescent probes, **TC1** and **TC2**, for specific imaging of lysosomes in living specimens (Fig. 1). **TC1** and **TC2** were designed by connecting the diphenylamine unit at the 4 and 4′-positions of the dithiophen-2-ylmethanone and ethyl 2-cyano-3,3-di(thiophen-2-yl)acrylate molecules (Scheme 1), respectively, in order to extend the π-conjugation of the final compounds and realize push–pull systems, which allow a fine control of the emission color of the dyes. The **TC** dyes exhibited excellent photostability, moderate quantum yield and large Stokes shift. Moreover, they were localized in lysosomes and showed high biocompatibility and long-term retention, important features for biological applications.

2. Materials and methods

2.1. Materials

All reactions were carried out under a nitrogen atmosphere. Solvents were freshly distilled prior to use, according to standard procedures. Commercial products were purchased from Sigma-Aldrich. LysoTracker Red DND-99 (**LTR**) and MitoTracker Red CMXRos (**MTR**) were purchased from Life Technologies. Glass bottom Petri dishes for confocal experiments were purchased from WillCo Dish®. The microwave used was a CEM Discover LabMate.

2.2. Measurements

UV–Vis absorption spectra were recorded on a Varian-Cary 500 spectrophotometer. Fluorescence spectra were recorded on a Varian Cary Eclipse spectrofluorimeter. Tris(bipyridine)ruthenium(II) chloride was used as the standard for determination of fluorescence quantum yields. Biological imaging tests were carried out with a Zeiss LSM700 (Zeiss, Germany) confocal microscope equipped with an Axio Observer Z1 (Zeiss, Germany) inverted microscope using an objective 100×, with 1.46 numerical aperture oil immersion lens for imaging. Laser beams with 405 nm, 488 nm and 542 nm excitation wavelengths were used for Hoechst, **TC1** and **TC2** dyes, and commercial trackers imaging, respectively.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer with chemical shifts reported in ppm ($CDCl_3$). LC–MS spectra were acquired with an Agilent 6300 Series Ion Trap interfaced to an Agilent 1200 HPLC adopting the following general conditions: atmospheric pressure chemical ionization, positive ions, chloroform as the eluent, flow rate 0.200 mL × min^{-1}, drying gas flow 5.0 L × min^{-1}, nebulizer pressure 60 psi, drying gas temperature 350 °C, vaporizer temperature 325 °C, and mass range 100–2200 m/z.

2.3. Synthetic procedures

2.3.1. Synthesis of compound *2*

To a stirred suspension of 5-bromothiophene-2-carboxylic acid **1** (5 g, 24.15 mmol) in CH_2Cl_2 (50 mL) oxalyl chloride (3.24 mL, 120 mmol) and N, N-dimethylformamide (10 μl) at 0 °C were added, and then the mixture was warmed at room temperature. After being stirred at the same temperature for 2 h, the solvent was evaporated under a reduced pressure to give the titled compound 5-bromothiophene-2-carbonyl chloride **2** (5.42 g), as a pale yellow solid, which was used in the subsequent step without further purification.

2.3.2. Synthesis of compound *3*

Under a N_2 atmosphere, a solution of **2** (5.42 g, 24 mmol) in anhydrous CH_2Cl_2 (35 mL) was added dropwise over a period of 5 min through a dropping funnel to a 250 mL three-necked round bottom flask containing a suspension of $AlCl_3$ (8.00 g, 60.37 mmol) in anhydrous CH_2Cl_2 (30 mL) under stirring. Then, a solution of 2-bromothiophene (4.00 g, 24.5 mmol) in 20 mL of anhydrous CH_2Cl_2 was added and the resulting mixture was stirred overnight at room temperature. The reaction mixture was poured into a mixture of ice (100 mL) and concentrated HCl (20 mL), then extracted with CH_2Cl_2. The organic phase was washed with brine, dried over anhydrous sodium sulfate and concentrated to dryness. Purification by column chromatography on silica gel (eluent: hexane:CH_2Cl_2, 8:2 v/v) yielded **3** as a yellow solid (50% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.59 (d, 2H, J = 4.1 Hz); 7.51 (d, 2H, J = 4.1 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 175.98, 143.37, 133.03, 131.02, 122.58.

2.3.3. Synthesis of *TC1*

Bis(5-bromothiophene-2-yl)methanone **3** (0.40 g, 1.13 mmol), diphenylamine (0.46 g, 2.70 mmol) and sodium tert-butoxide (0.29 g, 3.00 mmol) were added to a suspension of $Pd_2(dba)_3$ (0.06 g, 0.06 mmol) and P(*t*Bu)$_3$ (0.23 mL, 0.23 mmol, 1 M in toluene) in anhydrous and degassed toluene (5 mL), previously stirred under Ar for 10 min. The resulting solution was heated under microwave irradiation at a constant temperature of 110 °C for 50 min. The solvent was removed, and the residue was dissolved in CH_2Cl_2 and filtered off on a short celite column. The solvent was removed by rotary under a reduced pressure, and the residue was purified by column chromatography on silica gel (eluent hexane:CH_2Cl_2, 1:1) to yield the dye **TC1** as an orange solid (0.42 g, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ; 7.57 (d, 2H, J = 4.2 Hz), 7.34 (m, 10H), 7.27 (m, 6H), 7.21 (m, 4H), 6.34 (d, 2H, J = 4.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.62, 160.60, 146.47, 132.76, 131.15, 129.43, 125.02, 124.66, 114.16. MS (APCI): calcd. for $C_{33}H_{24}N_2OS_2$ 528.13; found: m/z = 529,1 $[M + H]^+$.

2.3.4. Synthesis of *TC2*

$TiCl_4$ (0.56 mL, 5.13 mmol) was slowly added into anhydrous $CHCl_3$ (9 mL) under ice bath and Ar atmosphere. After stirring for 10 min, **TC1** (0.1 g, 0.19 mmol) and ethyl 2-cyanoacetate (0.13 g, 1.17 mmol) were added to the reaction mixture. After 10 min pyridine (0.75 mL) was added into the mixture and heated to reflux under Ar for 10 h. Then, the mixture was quenched with water and then extracted with CH_2Cl_2.

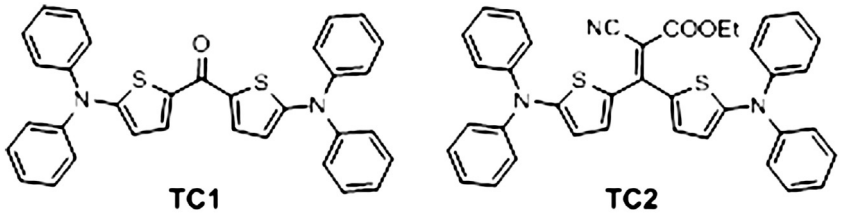

Fig. 1. Structure of **TC1** and **TC2** dyes.