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A new water-soluble polythiophene derivative as a probe for real-time monitoring adenosine 5′-triphosphatase activity in lysosome of living cells

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ABSTRACT

Detection of the adenosine 5′-triphosphatase (ATPase) activity in lysosome of living cells is of great importance for clinical diagnosis of many related diseases, including cancer. In this work, a new water-soluble polythiophene derivative named **ZnPT** bearing both quaternary ammonium salt groups and dipicolylamine-Zn^{2+} (DPA-Zn^{2+}) complexes in its side chain, was designed and synthesized for this propose. The probe mainly localized to lysosome with good biocompatibility and membrane penetration. The real-time, continuous, direct, and label-free assays were achieved through a fluorescence "turn-on" mode by taking advantages of the reaction specificity of ATPase with ATP and the high binding selectivity of **ZnPT** toward ATP substrate over its hydrolysis product (ADP). This well designed strategy should provide a facile and effective way for investigating ATPase-relevant biological processes.

1. Introduction

Adenosine 5′-triphosphatase (ATPase), is a class of enzymes that catalyze the decomposition of ATP into ADP and a free phosphate ion (Pi). This dephosphorylation reaction releases energy to drive most energy related cellular activity [1,2]. Detecting the activity of ATPase are essential for gaining a better understanding of critical processes involving ATPase in living systems [3,4]. Lysosome, membrane-bound organelle in mammalian cells, serves as a cellular recycling centre. Lysosome contains a vacuolar (H^+)-ATPase (V-ATPase), which can use the energy provided by ATP hydrolysis to pump H^+ in cytoplasm into lysosome to maintain its pH in range of 4.5–5.0 [5,6]. Abnormal V-ATPase activity have been found to be closely related to some human diseases, including cancer [7]. V-ATPase *in vivo* is generally integral membrane proteins (anchored within biological membranes) and is composed of the membrane sector and the cytoplasmic sector, which together determine its activity. Since it is difficult to directly detect V-ATPase activity in diseased cells at this stage, the development of a convenient real-time monitoring system for exogenous ATPase activity in lysosome of living cells would be highly desirable for the possible clinical diagnosis and treatment of related diseases [7,8].

Probes based on analytes-induced changes in fluorescence are favored due to their advantages of high sensitivity, fast response as well as high spatial and temporal resolution of fluorescence, facilitating *in vivo* and *in vitro* imaging [9–12]. Such systems possess enormous potential to reveal the pivotal roles that certain bioactive substrates have in dynamic cellular processes. Although there are some synthetic fluorescent probes for the detection of ATPase activity so far [13–15], they usually operate under physiological condition that mimics an extracellular fluid because these reported probes generally exhibit poor selectivity toward ATP substrate in complex environment. Therefore, it is still a challenging task to directly monitor ATPase activity in living cells using fluorescent probes as reporters.

In recent years, water-soluble fluorescent conjugated polymers (CPs) have been widely used as the optical platform for chemical and biological detection [16–23]. Among them, polythiophene (PT) derivatives [24,25] display unique changes in their conformation and aggregation states upon forming intermolecular complexes with a variety of biomolecules, such as DNA and protein [26,27], as well as small bioanions such as nucleotides [28–32], glutathione [33] and so on. Recently, some conjugated PT derivatives have been successfully used as optical probes for detection of the activity of some enzymes, such as protease [34], acetylcholinesterase [35] and hexokinase [36] in buffered solutions. In addition, it was noted that previously reported PT-based probes exhibited low toxicity and good photostability, and thus have been used as live-cell imaging materials [37–39]. So it is possible to use these properties of PTs for designing a simple, continuous and real-time protocol to assay the ATPase activity in living cells.

In the present work, a new water-soluble PT derivative named **ZnPT** was designed for the purpose of ATPase activity assays in living cells

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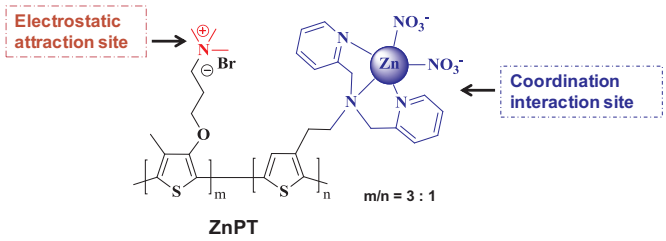

Scheme 1. The chemical structure of the polymer probe (**ZnPT**).

and the chemical structure was shown in Scheme 1. Both quaternary ammonium salt groups and dipicolylamine-Zn^{2+} (DPA-Zn^{2+}) complexes were introduced into the side chain of PT to provide a new probe with two possible recognition sites, *i.e.* electrostatic attraction site and coordination interaction site, respectively. **ZnPT** showed excellent selectivity toward ATP over other biological anions including ADP, AMP, calf thymus DNA (*ct*DNA) *etc.* due to the synergy of electrostatic attraction and coordination interaction between the probe and ATP. By taking the advantages of the reaction specificity of ATPase with ATP and the high binding selectivity of the probe toward ATP substrate over its hydrolysis product (ADP), the real-time assays of ATPase activity in living cells were achieved.

2. Experimental sections

2.1. Materials

ATPase, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, USA). LysoTracker Green and Mito-Tracker Green were obtained from Invitrogen (Shanghai, China) Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) were purchased from Aladdin Reagent Company (Shanghai, China). All other chemicals were local products of analytical grade and were used without further purification as otherwise indicated. All monomers were carefully purified prior for use in the polymerization reaction following the standard procedure. Ultrapure Millipore water (18.6 MΩ cm at 25 °C) was obtained with a Millipore filtration system. The polymer concentration is calculated based on the repeat units (RU).

2.2. Instruments

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV400 using residual solvent peak as a reference. High-resolution matrix-assisted laser desorption ionization (MALDI) mass spectra were collected with a Fourier transform-ion cyclotron resonance mass spectrometer instrument (Varian 7.0T FTICR-MS). GPC analysis was conducted with a Waters 2690 liquid chromatography system equipped with a Waters 996 photodiode detector and Phenogel GPC columns, using pullulan as the standard and H_2O as the eluent at a flow rate of 1.0 mL min^{-1} at 35 °C. 50 mL of 0.2 wt% of polymer in H_2O was injected into the columns. The UV–Vis absorption spectra were measured on a Shimadzu UV-2550 spectrophotometer. Fluorescence spectra of the samples were recorded by a Hitachi F-4600 spectrometer equipped with a xenon lamp excitation source. The pH measurement is carried out on a Mettler Toledo Delta 320 pH meter. Cells were imaged on a confocal microscope (Olympus FV1000-IX81). All images were analyzed with Olympus FV1000-ASW.

2.3. Synthesis and characterization

2.3.1. N, N-bis(pyridin-2-ylmethyl)-2-(thiophen-3-yl)ethanamine (1)

Monomer **1** was synthesized according to the similar procedure reported in the literature [40]. 3-(Aminoethyl)-thiophene (636 mg, 5.0 mmol), sodium bicarbonate (950 mg, 11.3 mmol) and sodium dodecyl sulfate (SDS) (28 mg, 0.1 mmol) were taken up in water (20 mL) and heated at 80 °C for 5 min 2-(Chloromethyl)pyridine (1.4 g, 11 mmol) was added to the reaction mixture and heated for 5 h at 80 °C. The reaction mixture was cooled to the room temperature and the mixture was extracted with ethyl acetate. The organic phase was dried with $MgSO_4$ and then filtrated, evaporated. The rude product was recrystallized from a mixture of ethyl acetate and hexane (1:3) to yield the pure product (601 mg, 38.8% yield). ^{1}H NMR (400 MHz, d_6-DMSO) δ: 8.47 (2H, dd), 7.71 (2H, td), 7.41 (3H, m), 7.23 (2H, dd), 7.11 (1H, d), 6.90 (1H, d), 3.80 (4H, s), 2.72 (2H, m), 2.69 (2H, t). HRMS: $C_{18}H_{19}N_3S$, calcd for $[M]^+$: 309.1300; found: 309.3437.

2.3.2. N, N, N-trimethyl-3-((4-methylthiophen-3-yl)oxy)propan-1-aminium bromide (2)

Monomer **2** was synthesized according to the procedure as reported previously by our group [30,32]. The characterization data for monomer **2** were shown as follows. ^{1}H NMR (400 MHz, D_2O) δ: 7.01 (d, 1 H), 6.48 (d, 1 H), 4.14 (m, 2 H), 3.53 (m, 2 H), 3.14 (s, 9 H), 2.30 (m, 2 H), 2.06 (s, 3 H). ^{13}C NMR (100 MHz, D_2O) δ: 154.73, 129.16, 120.77, 98.40, 66.72, 59.51, 56.38–51.18 (m), 22.64, 11.79. HRMS: $C_{11}H_{20}BrNOS$, calcd for $[M]^+$: 295.0414; found: 295.2672.

2.3.3. Synthesis of copolymer 3

The polythiophene derivative **3** was synthesized *via* oxidative polymerization by $FeCl_3$ according to the procedure reported in our previous work [30,32]. The monomer **1** (139.2 mg, 0.45 mmol), the monomer **2** (470.8 mg, 1.60 mmol) and anhydrous $FeCl_3$ were mixed in 25 mL of dry $CHCl_3$, and stirred for 48 h at room temperature under N_2 atmosphere. The reaction mixture was then evaporated to dryness. The solid was dissolved with methanol, and hydrazine hydrate was added to precipitate ferric chloride. The combined filtrate was evaporated under reduced pressure. The residue was dissolved in DMSO/H_2O (v/v 1:10). Then the solution was dialyzed through a membrane with a molecular weight cutoff of 3500 for three days and freeze-dried to yield the dark purple product **3** (351.6 mg, 57.6% yield).

2.3.4. Synthesis of ZnPT

ZnPT was prepared according to the similar procedure reported in the literatures [41,42]. $Zn(NO_3)_2{\cdot}6H_2O$ (372 mg, 1.25 mmol) was added into a solution of **3** (300 mg) in methanol. The mixture was stirred for 24 h at room temperature. After concentrating under reduced pressure, the excess $Zn(NO_3)_2{\cdot}6H_2O$ was removed by washed with methanol. The resulting polymer was dried under reduced pressure to give **ZnPT** (298 mg, 87% yield).

2.4. Cytotoxicity assay

The cytotoxicity of **ZnPT** against L929 cells were studied using a MTT cell-viability assay [39]. L929 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). Then, the cells were seeded into 96-well plates at a density of 7×10^3 cells in each well. After 24 h of incubation at 37 °C in a 5% CO_2 humidified atmosphere, the cells were treated with various concentrations of **ZnPT** aqueous solutions (0–50 μM), and cultured for another 24 h. After pouring out the medium, 100 μL of freshly prepared MTT (1 mg/mL in PBS) was added to each well and incubated for 4 h. After removing the MTT medium solution, the cells were lysed by adding 100 μL of DMSO. The plate was gently shaken for 5 min, and then the absorbance of purple formazan at 490 nm was monitored using a Spectra MAX 340PC plate reader.

2.5. Cellular imaging experiments

L929 cells were grown in RPMI 1640 supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C.