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A water-soluble fluorescent probe for the sensitive detection of endogenous alkaline phosphatase in living cells

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ABSTRACT

Alkaline phosphatase (ALP) is an essential enzyme in various tissues, which has been reported to be extensively involved in various physiological and pathological processes. Fluorescent probe assays carried out *in vivo* are desirable for monitoring the ALP activity. In this study, a novel red-light fluorescent DHP probe, which exhibited good selectivity and high sensitivity, for monitoring the ALP activity in real time was described. The DHP probe exhibited good water solubility, and the detection limit of ALP was determined as 0.031 U/L; this value is almost less than those of the other probes reported previously. The probe exhibited high selectivity, which will not interfere with various enzymes and amino acids. Moreover, the sensing and luminescence mechanisms of ALP to DHP was investigated by density functional theory (DFT) and time-dependent DFT. Furthermore, the DHP probe was applied for the detection of endogenous ALP in BEL-7402 cells, and the fluctuation of the ALP levels in BEL-7402 and HEK 293 T cells was successfully tracked by fluorescence microscopy.

1. Introduction

Alkaline phosphatases (ALP) constitute a subfamily of phosphatases, which catalyze the hydrolysis of phosphoryl esters and transphosphorylation of various substrates including proteins, phosphatides, nucleic acids, and inorganic phosphates [1,2]. ALP is expressed in various human tissues; particularly, it is concentrated in the liver, bone, kidney, and placenta [1,3]. Typically, serum ALP in human beings ranges from 46 to 190 mU/mL, and several diseases such as bone diseases, diabetes, liver dysfunction, breast and prostatic cancer, and hepatitis are associated with abnormal ALP levels [4–7]. Therefore, the sensitive detection of ALP is crucial to clinical diagnosis. Despite the extensive reported studies, the complicated physiological and pathological functions associated with the regulation of the ALP activity have not been completely defined. Moreover, it is still challenging to monitor the real-time ALP activity *in vivo* because of the absence of appropriate tools to a certain extent. Thus far, few assay techniques for the ALP activity have been reported, including isotopic labeling [8], surface-enhanced resonance Raman scattering [9], electrochemistry [10], chromatography [19], colorimetry [11], and chemiluminescence [12]. Among different methods for detecting the ALP activity, fluorescence appears to be an ideal choice because of its high sensitivity and rapid analysis, especially as a nondestructive, noninvasive method for providing target information of living cells [13,14]. Thus far, various fluorescent biosensors have been successfully reported to detect the ALP activity on the basis of inorganic semiconductor quantum dots (QDs) [15], carbon QDs [16], and organic fluorescent dyes [17]. However, these methods exhibit disadvantages such as low sensitivity of the polymer and the high toxicity of QDs. By contrast, organic fluorescent probes exhibit key advantages of cost-effectiveness, nontoxicity, and facile operation. The majority of the probes cannot used for deep-tissue imaging because their Ex/Em wavelengths are extremely short to cause high background interference [18]. Therefore, it is important to develop a new fluorescent probe with emission wavelengths in the red light to NIR region.

In this study, a novel water-soluble fluorescent probe for the highly sensitive and selective detection of ALP was developed. Scheme 1 shows the chemical structure. The DHP probe was designed by the addition of phosphate groups to the fluorescent dye with an emission wavelength in the red-light region. Generally, an ICT system comprises electron-donating and electron-withdrawing groups, and ICT occurs from the donor to acceptor on photoexcitation. However, DHP exhibits

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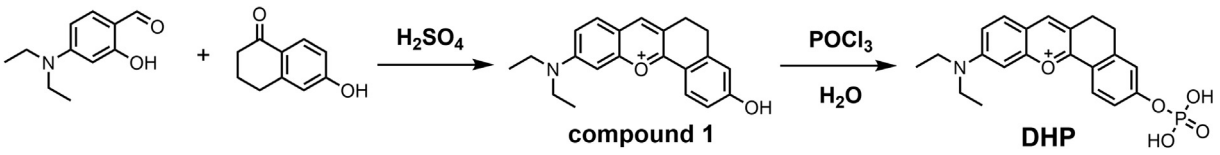

Scheme 1. Synthesis of DHP.

good water solubility, which is different from that of small-molecule probes. With the addition of ALP to a buffered aqueous solution, remarkable fluorescence was observed at 615 nm, with a 17-fold enhancement. DHP exhibited a low detection limit of 0.031 U/L and low cytotoxicity. In addition, the application of DHP to investigate the endogenous ALP activity in cells was discussed.

2. Experimental section

2.1. Chemicals and instrumentation

4-(Diethylamino)salicylaldehyde, 6-hydroxy-1-tetralone, pyridine, and sodium orthovanadate (Na_3VO_4) were purchased from Aladdin Chemistry Co., Ltd (Shanghai, China). ALP, acid phosphatase (ACP), cysteine (Cys), glutathione (GSH), and acetylcholinesterase (AChE) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. Bovine serum albumin (BSA) was purchased from Ding Guo Biotechnology Company. Horse IgG was purchased from Beijing Biosynthesis Biotechnology Company. Unless noted, analytical-reagent grade chemicals were used without further purification. All aqueous solutions were prepared using ultrapure water obtained from a Milli-Q water purification system (18.2 MΩ cm).

^{1}H NMR and ^{13}C NMR spectra were recorded on a Mercury 300 B B nuclear magnetic resonance spectrometer (Varian Inc., USA) at 300 and 75 MHz, respectively. ^{31}P NMR spectra were recorded on Avance III500 NMR spectrometer (Bruker Inc., Germany) at 243 MHz. Mass spectra (MS) were recorded on a TSQ Quantum Access MAX triple-quadrupole mass spectrometer (Thermo Fisher Scientific, USA). pH was measured on an INESA Scientific PHS-3C pH meter. Fluorescence spectra were recorded on an F-2700 fluorescence spectrometer (Hitachi Co., Ltd. Japan) with a 1-cm quartz cell. Absorption spectra were recorded on a Cary 60 UV-spectrophotometer (Agilent Technologies, USA) with a 1-cm quartz cell. Cell imaging experiments were carried out on an LSM 710 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

2.2. Synthesis

2.2.1. Synthesis of compound *1*

First, 6-hydroxy-1-tetralone (0.6 g, 2.7 mmol) was slowly added into concentrated H_2SO_4 (1.85 mL), followed by cooling to 0 °C. Second, 4-(diethylamino)salicylaldehyde (0.784 g, 4.06 mmol) was added in portions over 1 h under vigorous stirring. Next, the reaction mixture was heated to 90 °C for 6 h. The resulting mixture was poured into ice (40 g) after cooling to room temperature. The mixture was stirred for 20 min, followed by extraction using CH_2Cl_2/CH_3OH (7 × 20 mL, v/v 10:1). The crude product was purified by silica-gel column chromatography using CH_2Cl_2/CH_3OH as the eluent, affording the final product in 68% yield (0.588 g). ^{1}H NMR (300 Hz, DMSO-d_6): δ 8.42 (2H, s), 8.10 (2H, d), 7.81 (2H, d), 7.29 (2H, m), 7.18 (2H, s), 6.81 (2H, m), 6.72 (2H, d), 3.63 (8H, q), 2.96 (8H, s), 1.22 (13H, t). ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.27, 157.71, 154.77, 147.80, 145.68, 131.49, 128.88, 120.17, 117.45, 117.15, 115.66, 45.21, 26.48, 24.61, 12.37. MS (ESI, *m/z*) Calcd for $[C_{21}H_{22}NO_2]^+$: 320.1645, found: 320.1960.

2.2.2. Synthesis of DHP compound

First, compound **1** (0.04 g, 0.125 mmol) was dissolved in anhydrous pyridine (4 mL), followed by the dropwise addition of phosphorus oxychloride (0.05 mL) to the reaction mixture. The mixture was stirred for 4 h at room temperature, and the resulting solution was poured into ice (15 g) and stirred overnight. Then, the solvent was removed, and the product was purified by silica-gel chromatography using 5:1 CH_2Cl_2/CH_3OH (0.0273 g, 54.5%). ^{1}H NMR (300 Hz, DMSO-d_6): δ 8.66 (1H, s), 8.58 (1H, d), 8.16 (1H, d), 7.92 (1H, d), 7.83–7.75 (1H, m), 7.39 (2H, m), 7.31 (2H, m), 3.68 (4H, d), 3.03 (4H, s), 1.24 (6H, t). ^{13}C NMR (75 MHz, CD_3OD) δ 165.15, 164.54, 160.25, 157.47, 146.01, 129.02, 122.04, 121.49, 121.42, 121.26, 121.19, 120.04, 119.19, 47.19, 28.19, 26.36, 12.92. ^{31}P NMR (243 MHz, DMSO-d_6) δ -0.39. MS (ESI, *m/z*) Calcd for $[C_{21}H_{23}NO_5P]^+$: 400.1308, found: 400.0281.

2.3. Spectral measurement

Fluorescence and absorption spectra were recorded according to the following procedure. First, the stock solution of DHP (20 μM) was prepared in a Tris-HCl buffer solution (25 mM, pH = 8.0). Various concentrations of ALP solutions were prepared by the dissolution of ALP in the Tris-HCl buffer solution (25 mM, pH = 8.0) with $MgCl_2$ (200 μM). Then, 0.5 mL of the DHP stock solution and 0.5 mL of the ALP stock solution were mixed to obtain a 1.0 mL test solution containing DHP (10 μM), $MgCl_2$ (100 μM), and 25 mM Tris-HCl. The resulting solutions were maintained at 37 °C for 15 min, and spectral properties were measured. Fluorescence emission spectra were recorded at an excitation of 570 nm and emissions ranging from 580 to 700 nm.

The cytotoxic effects of DHP were examined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. BEL-7402 cells seeded in a 96-well plate were incubated with a series of DHP concentrations (0, 1.25, 2.5, 5, 10, and 20 μM). After the cells were incubated at 37 °C for 24 h, the MTT solution was added into each well, and the residual MTT solution was removed after 4 h, followed by the addition of DMSO to each well to dissolve the formazan crystals. After shaking for 10 min, the absorbance at 490 nm was recorded using a microplate reader.

2.4. Cell culture and fluorescence imaging

BEL-7402 and HEK 293 T cells were treated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2. The cells were then plated on a 35-mm culture dish and allowed to adhere for 24 h.

First, BEL-7402 cells were incubated with DHP (10 μM) at 37 °C for 15 min after washing three times with the PBS buffer solution. Second, the cells were incubated with Na_3VO_4 (100 μM) for 30 min, followed by incubation with DHP (10 μM) for another 15 min. After each step, washing with the PBS buffer solution (pH = 7.4) was carried out. Similar steps carried out for the treatment of BEL-7402 cells were performed for HEK 293 T cells. All cells in the experiments were washed three times with PBS before imaging.

3. Results and discussion

3.1. Synthesis and the response mechanism

In this study, a novel compound (Scheme 1) with emission wavelength in the red-light region was successfully designed and synthesized. This compound is quenched because of the modification of the hydroxyl group on the dye to phosphate groups with phosphorus