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Construction of an alkaline phosphatase-specific two-photon probe and its imaging application in living cells and tissues

Huatang Zhang [a, b], Peng Xiao [c], Yin Ting Wong [d], Wei Shen [b], Mohit Chhabra [b], Raoul Peltier [b], Yin Jiang [a], Yonghe He [a], Jun He [a], Yi Tan [b], Yusheng Xie [b], Derek Ho [e], Yun-Wah Lam [b, *], Jinpeng Sun [c, **], Hongyan Sun [b, ***]

[a] School of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou, Guangdong, 510006, PR China
[b] Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, China
[c] Department of Physiology, Shandong University School of Medicine, Jinan, Shandong, 250012, PR China
[d] Department of Biomedical Sciences, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, China
[e] Department of Physics and Materials Science, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, China

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ABSTRACT

Alkaline phosphatase (ALP) is a family of enzymes involved in the regulation of important biological processes such as cell differentiation and bone mineralization. Monitoring the activity of ALP in serum can help diagnose a variety of diseases including bone and liver diseases. There has been growing interest in developing new chemical tools for monitoring ALP activity in living systems. Such tools will help further delineate the roles of ALP in biological and pathological processes. Previously reported fluorescent probes has a number of disadvantages that limit their application, such as poor selectivity and short-wavelength excitation. In this work, we report a new two-photon fluorescent probe (TP-Phos) to selectively detect ALP activity. The probe is composed of a two-photon fluorophore, a phosphate recognition moiety, and a self-cleavable adaptor. It offers a number of advantages over previously reported probes, such as fast reaction kinetics, high sensitivity and low cytotoxicity. Experimental results also showed that TP-Phos displayed improved selectivity over DIFMUP, a commonly utilized ALP probe. The selectivity is attributed to the utilization of an ortho-functionalised phenyl phosphate group, which increases the steric hindrance of the probe and the active site of phosphatases. Moreover, the two-photon nature of the probe confers enhanced imaging properties such as increased penetration depth and lower tissue autofluorescence. TP-Phos was successfully used to image the endogenous ALP activity of hippocampus, kidney and liver tissues from rat.

1. Introduction

Alkaline phosphatase (ALP) is a family of enzymes involved in the regulation of phosphate metabolism in both prokaryotes and eukaryotes. ALP can act on a variety of biomolecules, including proteins, phosphatidates, nucleic acids and inorganic phosphates. By catalyzing the hydrolysis and transphosphorylation of monophosphate esters at alkaline pH, ALP is involved in regulating many important biological processes, including cell differentiation, bone mineralization and detoxification of bacteria endotoxin [1,2]. Studies have also shown that abnormal levels of ALP is associated with a variety of human diseases, such as bone diseases [3,4], liver dysfunction [5,6], breast and prostatic cancer [7,8], and diabetes [9,10]. Despite these advancements in research, the diverse physiological and pathological functions of ALP still remain largely unknown. Consequently there has been growing interest in developing chemical tools for accurate and selective detection of ALP activity in living systems. Such tools will help further delineating the roles of ALP in various biological and pathological processes. Furthermore, developing chemical tools for sensing ALP might find useful applications in clinical diagnosis.

Among different methods to detect ALP activity, the fluorescent probe approach has proven to be particularly useful, especially as a

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: yunwlam@cityu.edu.hk (Y.-W. Lam), sunjinpeng@sdu.edu.cn (J. Sun), hongysun@cityu.edu.hk (H. Sun).

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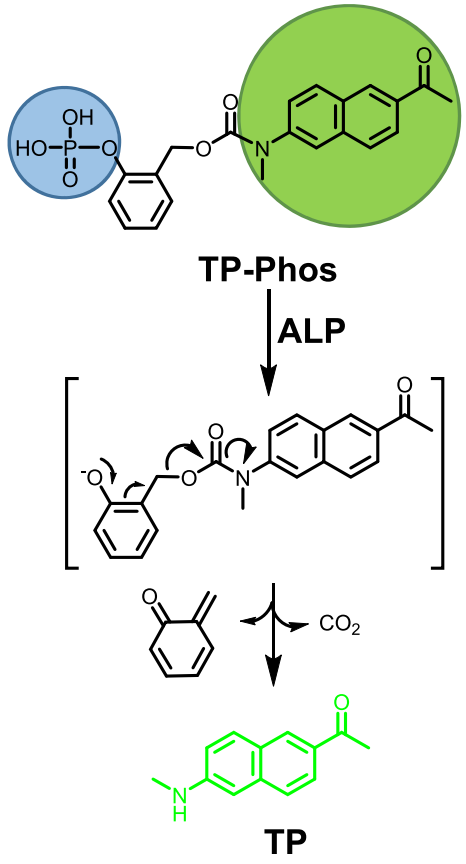
nondestructive and noninvasive way to monitor the biological targets in real time. To date, a number of fluorescent probes have been reported to be able to detect ALP activity. These probes are mainly based on small-molecule fluorophores or inorganic nanomaterials [11–17]. However, the vast majority of the currently available fluorescent probes perform at short excitation wavelengths, usually in the UV–vis range. As a result, they have shallow tissue penetration depths and their application in tissue imaging is limited. In addition, most of the fluorescent probes cannot differentiate ALP from other phosphatases, such as protein tyrosine phosphatases (PTPs), resulting in poor selectivity of the probes. For example, commercially available ALP fluorescent probes such as MUP [18,19], DiFMUP [20–22], and FDP [23–25] show similar reactivity towards ALP and other phosphatases.

To overcome the aforementioned constraints, we herein report the ALP specific fluorescent probe with two-photon properties and its imaging application in cells and tissues. The probe TP-Phos consists of three parts: a two-photon (TP) fluorophore, a phosphate recognition group, and a self-cleavable linker (Scheme 1 and Scheme 2). We hypothesize the "turn on" mechanism of the probe as follows (Scheme 1). ALP first catalyzes the dephosphorylation of TP-Phos to produce a phenolate intermediate. Subsequently the intermediate undergoes 1,4-elimination reaction and releases ortho-quinone methide, leading to the "turn on" of fluorescence [26,27]. Compared with the traditional one-photon fluorophore, our TP probe has a number of advantages, including increased penetration depth, low tissue auto-fluorescence and self-absorption, high resolution, reduced photo damage and photo bleaching [28,29]. Furthermore, we specifically incorporated an ortho-functionalised phenyl phosphate group in the probe design to increase the steric hindrance between the phosphate recognition group and the active site of phosphatase. This allows ALP to be differentiated from other phosphatases. Lastly, we found that the probe showed excellent stability in DMSO and did not show any obvious decomposition after being stored at −20 °C for two years (Fig. S1).



Scheme 1. Chemical structure of TP-Phos probe and its proposed "turn-on" mechanism by ALP.

2. Material and methods

2.1. Materials and general instruments

All chemicals and solvents were purchased from commercial suppliers with the highest grade. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA and penicillin/streptomycin were purchased from Invitrogen. Calf intestinal alkaline phosphatase was purchased from NEB. The BIOMOL Green™ Reagent for Phosphate Detection, BML-AK111, was purchased from Enzo Life Sciences. RIPA lysis buffer and protease inhibitor cocktail were purchased from Sigma-Aldrich. 6, 8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) was purchased from Sangon Biotech Co. Ltd. 4-methylumbelliferyl phosphate (4-MUP) was purchased from J&K Chemical Ltd. Other chemicals were used directly in the experiment without further purification, unless otherwise specified. Ibidi® 8-well culture plates were purchased from ibidi GmbH. C57BL/6 mice were obtained from the Laboratory Animal Services Center of the Chinese University of Hong Kong, and all experiments were performed in accordance with local guidelines. Fresh tissue slices were prepared with Leica VT1000S. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were recorded on a Bruker NMR spectrometer (400 MHz or 300 MHz). Mass spectra were obtained on a PC Sciex API 150 EX ESI-MS system using electrospray ionization (ESI). UV absorption spectra were obtained on Shimadzu 1700 UV/Vis Spectrometer. Fluorescence signal was recorded with a FluoroMax-4 fluorescence photometer. Fluorescence images were acquired using a Leica TCS SP5 Confocal Scanning Microscope. pH value was recorded with a FiveEasy ™ Fe20 pH meter.

2.2. Synthesis

2.2.1. Synthesis of diethyl (2-formylphenyl) phosphate (compound A)

2-hydroxybenzaldehyde (2 mmol, 244 mg) and K_2CO_3 (10 mmol, 1.38 g) were dissolved in THF (20 mL). Diethyl phosphorochloridate (3 mmol, 432 μL) was then added to the solution and stirred at room temperature for 6 h. The organic solvent was evaporated. CH_2Cl_2 and water were subsequently added for extraction. CH_2Cl_2 phase was collected and dried with $MgSO_4$. The crude product was purified by silica gel column chromatography. Compound A was obtained with a yield of 91%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.42 (s, 1H), 7.90 (d, J = 8.0 Hz, 1H), 7.61 (t, J = 8.0 Hz, 1H), 7.48 (d, J = 8.0 Hz, 1H,), 7.31 (t, J = 8.0 Hz, 1H), 4.26 (t, J = 7.2 Hz, 4H), 1.37 (t, J = 7.2 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) 188.5, 152.8, 135.7, 128.8, 127.3, 125.4, 121.1, 65.1, 16.1; ^{31}P NMR ($CDCl_3$, 162 MHz) δ −6.67. ESI-MS: Calcd. for $C_{11}H_{16}O_5P$ $[M+H]^+$ 259.1; found 259.4.

2.2.2. Synthesis of diethyl (2-(hydroxymethyl)phenyl) phosphate (compound B)

Compound A (1 mmol, 258 mg) was dissolved in 20 mL of mixed solvent (E_tOH/THF/H_2O = 100/20/1, v/v/v) and cooled in an ice bath. $NaBH_4$ (2 mmol, 76 mg) was added to the above solution. The reaction mixture was stirred at room temperature for 1 h. The solvent was then removed. 1 mol/L aqueous HCl was added to the residue and extracted with ethyl acetate. Organic phase was evaporated and purified with silica gel column chromatography (ethyl acetate/Hexane, from 1/5 to 2/1). Compound B was obtained with a yield of