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Fluorescent Light-up Probe with Aggregation-Induced Emission Characteristics for Alkaline Phosphatase Sensing and Activity Study

Jing Liang,[†,‡] Ryan Tsz Kin Kwok,[‡,§] Haibin Shi,[†] Ben Zhong Tang,[*,§,⊥] and Bin Liu[*,†,#]

[†]Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive, Singapore 117576

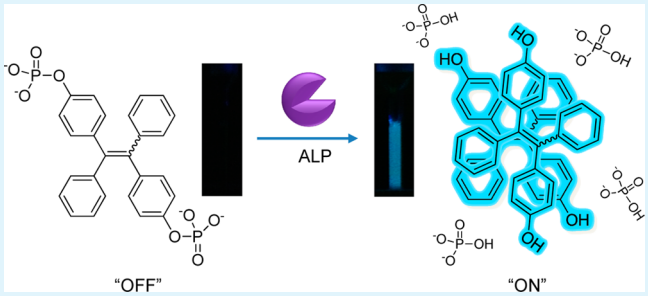
[§]Department of Chemistry, Division of Biomedical Engineering, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

[⊥]SCUT-HKUST Joint Research Laboratory, Guangdong Innovative Research Team, State Key Laboratory of Luminescent Materials and Devices, South China University of Technology, Guangzhou 510640, China

[#]Institute of Materials Research Engineering, 3 Research Link, Singapore 117602

Supporting Information

ABSTRACT: Fluorogens with aggregation-induced emission (AIE) characteristics have attracted intensified research interest in biosensing applications, and those with specific targeting ability are especially desirable. In this work, we designed and synthesized an AIE fluorescent probe by functionalizing a tetraphenylethylene (TPE) fluorogen with two phosphate groups (TPE-phos) for the detection of alkaline phosphatase (ALP) and its enzymatic activity based on the specific interaction between the probe and ALP. The probe is virtually nonfluorescent in aqueous media due to good water solubility. In the presence of ALP, the phosphate groups are cleaved through enzymatic hydrolysis, yielding a highly fluorescent product as a result of activated AIE process. This light-up probe shows excellent selectivity toward ALP among a group of proteins. The detection limit is found to be 11.4 pM or 0.2 U L^{-1} in Tris buffer solution with a linear quantification range of 3−526 U L^{-1}. The assay is also successfully performed in diluted serum with a linear range up to 175 U L^{-1}, demonstrating its potential application in clinical analysis of ALP levels in real samples. Furthermore, by conducting kinetic analysis of the enzyme using TPE-phos as the substrate, the kinetic parameter k_{cat}/K_M is determined to be 5.1×10^5 M^{-1} s^{-1}, indicating a high efficiency of the substrate.



KEYWORDS: *aggregation-induced emission, AIE fluorogen, light-up probe, alkaline phosphatase (ALP), enzyme activity, protein detection*

INTRODUCTION

Alkaline phosphatase (ALP), a class of enzyme found in various sources of mammals (bone, liver, placental, and intestinal), has long been identified as an important biomarker for clinical diagnostics.[1] Excessive level of ALP in the blood serum is often correlated with bone, liver dysfunction, heart failure, ovarian and breast cancer, leukemia and so on.[2−4] On the basis of its ability to remove phosphate groups from a wide variety of chemicals, a number of strategies have been established for ALP detection.[5−10] Among them, fluorescence detection has become extremely popular due to its rapid, sensitive signal transduction. A few fluorometric assays have been reported based on conjugated polyelectrolyte,[11,12] quantum dots,[13,14] and organic fluorophores[15] for the detection of ALP activities. However, they all require an additional quencher moiety or multiple steps to enable fluorescence signaling. Fluorogenic substrates have also been developed for ALP activity study with direct fluorescence turn-on signals upon cleavage by ALP;[7,16] however, these probes generally show high fluorescence background. ALP assays combining simplicity, high sensitivity, and selectivity remain attractive.

Fluorogens with aggregation-induced emission (AIE) characteristics have recently emerged as a novel class of materials for biosensing and imaging applications.[17] Unlike conventional fluorophores that suffer from fluorescence quenching in high concentration or solid state due to $\pi-\pi$ stacking interaction,[18] AIE fluorogens like tetraphenylethylene (TPE) and siloles derivatives experience increased emission when they are aggregated. Because of their unique propeller-like structure, their intramolecular rotations are restricted in aggregated state, which prohibits energy dissipation via nonradiative channels, leading to high quantum yields.[19] A number of AIE-based enzyme assays have been reported for nuclease,[20] phosphatase[21] and acetylcholinesterase[22,23] detection. However, the majority of the assays adopt fluorescence turn-off scheme and

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mainly rely on electrostatic or hydrophobic interactions between AIE fluorogens and the substrate, which lacks specificity and can be easily disturbed by external stimuli. Fluorescence turn-on enzyme assays have also been reported, but they generally require multiple steps or chemical reactions to achieve fluorescence light-up.[24,25] Although recent work has also reported an ALP light-up probe based on a phosphate-functionalized TPE fluorogen,[26] the probe shows obvious background signal in aqueous media, which compromises the detection sensitivity.

Herein, we report a one-step light-up assay for ALP relying on dephosphorylation of the phosphate-bearing TPE (TPE-phos) by ALP to produce a highly fluorescent residue. This molecular probe is hypothesized to have a few advantages. First, the probe possesses a simple structure with easy synthetic steps and modification; second, the probe has negligible fluorescence signal in aqueous medium so that a high signal-to-background ratio can be achieved; third, the cleaved product is insoluble in the reaction medium which favors the reaction equilibrium. Taking advantage of the enhanced fluorescence, a specific light-up probe for ALP sensing and enzyme activity studies is constructed. In addition, the assay works well in serum, which further expands its potential application in clinical applications. The kinetic behavior of ALP is also studied using TPE-phos as the substrate and kinetic parameters are determined.

EXPERIMENTAL SECTION

Chemicals and Instruments. Phosphatase alkaline (ALP) from bovine intestinal mucosa, bovine serum albumin (BSA), lysozyme, trypsin, pepsin, papain and 4-methylumbelliferyl phosphate (4-MUP) were purchased from Sigma. Tetrahydrofuran (THF) was distilled from sodium benzophenoneketyl immediately prior to use. Dichloromethane (DCM) and chloroform were distilled over calcium hydride. Inhibitor ethylenediaminetetraacetic acid disodium salt (EDTA) was purchased from AnalaR. Tris buffer (1 M, pH 8.0) was purchased from 1st Base. Fetal bovine serum (FBS) was purchased from Thermo Scientific. Triethylamine was purchased from sigma-aldrich and was distilled before use. Dimethyl sulfoxide (DMSO), and iodotrimethylsilane (TMS-I), 4-hydroxylbenzophenone, zinc dust, diethyl chlorophosphate, and trimethylsilyl iodide were all purchased from Sigma and used as received without further purification.

The UV−vis absorption spectra were obtained using UV−vis spectrometer (Shimadzu, UV-1700, Japan). Photoluminescence (PL) measurements were carried out on a Perkin-Elmer LS-55 equipped with a xenon lamp excitation source and a Hamamatsu (Japan) 928 PMT, using 90° angle detection for solution samples. The zeta potential measurements were done using a zeta potential analyzer (ZetaPlus, Brookhaven Instruments Corporation) at room temperature. The average particle size and size distribution of TPE-phos and TPE-2OH were determined by laser light scattering (LLS) with particle size analyzer (90 Plus, Brookhaven Instruments Co. USA) at a fixed angle of 90° at room temperature. The cells were imaged by fluorescence microscope (Nikon A1 Confocal microscope). The time-dependence fluorescence scans of TPE-phos were conducted with microplate reader (TECAN). High-resolution mass spectra (HRMS) were recorded on a Finnigan TSQ 7000 triple-quardrupole mass spectrometer operating in a chemical ionization (CI) mode with methane as carrier gas. ^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 600 NMR spectrometer. Chemical shifts were reported in parts per million (ppm) referenced with respect to residual solvent ($CDCl_3$ = 7.26 ppm, $(CD_3)_2SO$ = 2.50 ppm or tetramethylsilane $Si(CH_3)_4$ = 0 ppm). The synthesized TPE-phos probe was purified by preparative high pressure liquid chromatography (HPLC) (Agilent 1100 series). The analytical LC profiles and molecular mass were acquired using liquid chromatography-ion trap-time-of-flight mass spectrometry (LCMS-IT-TOF) (Shimadzu). 0.1% TFA/H_2O and 0.1% TFA/acetonitrile were used as eluents for all HPLC experiments. The flow rate was 0.6 mL/min for analytical HPLC and 2 mL/min for preparative HPLC.

Synthesis of Compound 2 (TPE-2OH). Into a 100 mL round-bottom flask with condenser, a mixture of 4-hydroxylbenzophenone (0.4955 g, 2.5 mmol) and zinc dust (0.4053 g, 6.2 mmol) in freshly distilled THF (60 mL) was cooled to −78 °C under N_2. $TiCl_4$ (6.2 mL, 6.2 mmol) was added dropwise to the cold mixture. The suspension was warmed to room temperature and then refluxed overnight. After warming to room temperature, the excess zinc residue was removed by flash chromatography using THF as solvent and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography using petroleum ether/chloroform (1:3) to obtain compound **3**, which have been used in next step immediately because of instability of the compound. ^{1}H NMR (400 MHz, $CDCl_3$), δ (TMS, ppm): 7.118−7.062 (m, 6H), 7.048−6.994 (m, 4H), 6.908−6.856 (m, 4H), 6.592−6.538 (m, 4H). Yield: 64%.

Synthesis of Compound 3. A mixture of **2** (0.3451 g, 1.0 mmol), freshly distilled triethylamine (0.70 mL, 5.0 mmol) and chlorophosphoric acid diethyl ester (0.29 mL, 2 mmol) in dry chloroform (4.0 mL) was stirred at room temperature under N_2 overnight. The reaction mixture was then concentrated under reduced pressure. The residue was purified on silica gel with petroleum ether/ethyl acetate (1:1) to afford compound **3**. ^{1}H NMR (400 MHz, $CDCl_3$), δ (TMS, ppm): 7.115−7.083 (m, 6H), 7.009−6.929 (m, 12H), 4.208−4.097 (m, 8H), 1.363−1.291 (m, 12H). Yield: 68%. HR-MS (MALDI-TOF): m/z 636.2010 [$(M)^+$, calcd 636.2042].

Synthesis of Compound 4 (TPE-phos). Compound **3** (100 mg) is dissolved in DCM (5 mL) in the round-bottom flask. 110 μL of TMS-I was added slowly into **3** using syringe while stirring. The reaction mixture was tightly sealed and allowed to react for ∼3 h at room temperature. The solvent was partially removed using rotary evaporator and the concentrated crude product was purified using preparative HPLC. Yield: 36%.^{1}H NMR (600 MHz, DMSO-d_6), δ (TMS, ppm): 7.17−7.10 (m, 6H), 6.99−6.98 (m, 4H), 6.90 (s, 8H), 6.48 (br s, 4H). ^{13}C NMR (125 MHz, DMSO-d_6), δ (TMS, ppm): 149.92, 149.88, 143.2, 139.7, 138.7, 131.7, 130.6, 127.9, 126.6, 119.22, 119.19. HR-MS (MALDI-TOF): m/z 524.0972 [$(M)^+$, calcd 524.0790].

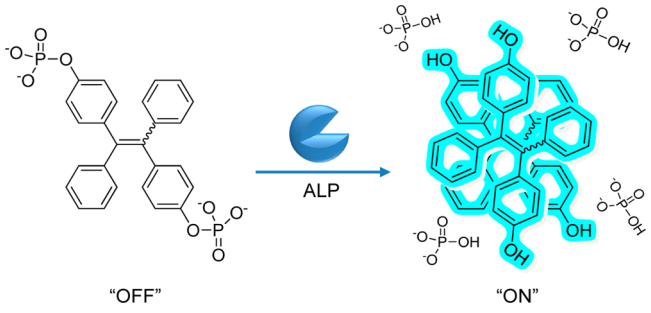
General Procedures for Enzymatic Assay. TPE-phos stock solution (0.5 μL, 1 mM in DMSO) was diluted with Tris buffer (10 mM, pH 8.0) to make 10 μM working solution. 0.4 μL of ALP stock solution (2.5 μM in Tris buffer, 10 mM, pH 8.0, 2 mM $MgCl_2$, 0.2 mM $ZnCl_2$) was added to the working solution. The mixture was incubated for 20 min at room temperature (23 °C). The solution was measured for fluorescence intensity using microplate reader. The emission intensity was recorded at 450 nm upon excitation of 312 nm. For fluorescence change versus incubation time, the fluorescence was scanned in 30 s intervals. For PL measurement, the solution was further diluted to 500 μL with Milli-Q water. The emission was recorded from 360 to 580 nm at excitation of 312 nm.

RESULTS AND DISCUSSION

As illustrated in Scheme 1. The probe molecule consists of a TPE terminally functionalized with two phosphate groups. The phosphate groups not only serve as specific substrate for ALP, but also convert the probe into a highly water-soluble species to render it almost nonfluorescent in water. In the presence of the ALP, the phosphate groups are recognized and cleaved to yield insoluble TPE residue with two hydroxyl groups (TPE-2OH). The formation of TPE-2OH nanoaggregates in water is highly emissive because of activation of the restriction of intramolecular rotations (RIR) mechanism.[19]

The TPE-phos probe was synthesized in three steps as shown in the synthetic scheme in Scheme 2. The final product TPE-phos was purified using HPLC and further characterized by NMR (see Figure S1 in the Supporting Information) and LCMS-IT-TOF (Figure S2) in the Supporting Information.

Scheme 1. Illustration of Design Principle of ALP Assay



The absorption and emission properties of both TPE-2OH and TPE-phos are examined in DMSO/water (v/v = 1:99). Figure 1 shows the UV–vis absorption and PL spectra of both chemicals at absorption maxima at 312 and 307 nm, respectively, with a similar absorbance. As expected, TPE-phos is virtually nonfluorescent in DMSO/water mixture, with a quantum yield (Φ) of 0.05% using quinine sulfate as reference; while TPE-2OH emits strong fluorescence in the same medium, giving a bright greenish blue color with an emission maximum at ~480 nm (Φ = 4.92%). The enhanced emission is associated with the formation of TPE-2OH aggregates, which is supported by laser light scattering (LLS) measurements. The effective diameter of TPE-2OH in the media is 106 ± 1 nm (Figure 2), whereas no LLS signal was collected for TPE-phos, suggesting its well-solubilized state.

On the basis of the distinct emission properties of TPE-2OH and TPE-phos, it is possible to construct an assay for ALP by monitoring the fluorescence changes after addition of ALP to the probe. Figure 3 shows the PL intensity profiles of the probe TPE-phos (10 μM) incubated for 20 min in the absence and presence of ALP (20 nM) in Tris reaction buffer (10 mM, pH 8.0). The fluorescence intensity of the probe incubated with ALP significantly increases as compared to that without ALP, with a 105-fold enhancement at the emission maximum. As activity of ALP from bovine intestinal mucosa requires the presence of metal ion cofactors (Cu^{2+}, Mg^{2+}), metal chelator such as ethylenediaminetetraacetic acid (EDTA) can effectively inhibit ALP activity by depleting metal ions in the reaction buffer.[27] To confirm the specific interaction between the probe and ALP, EDTA (0.5 mM) was incubated with ALP for 20 min before incubation with the probe. It is observed that the fluorescence of the TPE-phos with EDTA is as low as that of the bare probe, indicating that the activity of ALP is almost completely inhibited. Because both the probe (zeta potential = −10.4 mV) and the enzyme (pK_a ≈ pH 5.4) are negatively charged at pH 8.0, the electrostatic interaction between the two

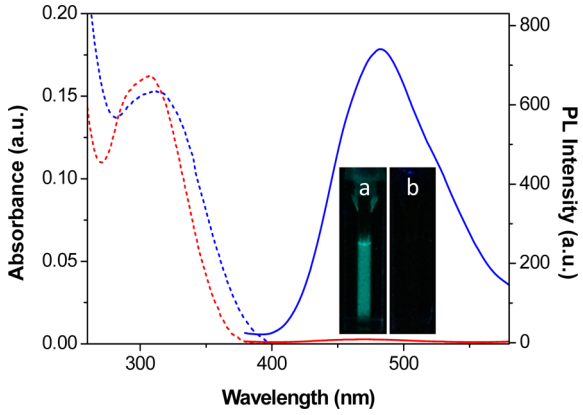


Figure 1. UV–vis absorption (dashed line) and PL (solid line) spectra of 10 μM TPE-2OH (blue) and TPE-phos (red) in DMSO/water (1:99 v/v). λ_{ex} = 312 nm. The inset shows the photographs of (a) TPE-2OH and (b) TPE-phos under UV lamp illumination (λ_{ex} = 365 nm).

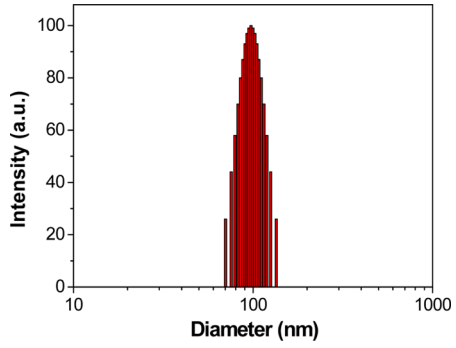
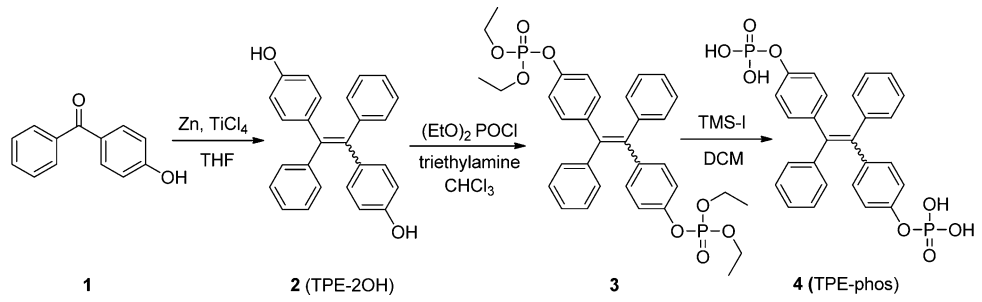


Figure 2. Hydrodynamic diameters of TPE-2OH in DMSO/H_2O (v/v = 1:99) measured by LLS.

should be minimum. Hence the mechanism of the fluorescence turn-on response is mainly attributed to the specific interaction between the probe and ALP that leads to the cleavage of the weakly fluorescent TPE-phos into its highly fluorescent counterpart TPE-2OH.

The emission of AIE fluorogen can be revitalized upon restriction of intramolecular rotations, which is possible through both self-aggregation and binding with other molecular species, such as the hydrophobic pockets of proteins. LLS measurements results found that strong signals were collected for TPE-phos only after incubation with ALP and the particle size is ~184.4 ± 12 nm after 15 min of incubation. The emission properties of the hydrolyzed product TPE-2OH in DMSO/Tris buffer solution with different volume fractions (0–

Scheme 2. Synthetic Route for the Probe TPE-phos



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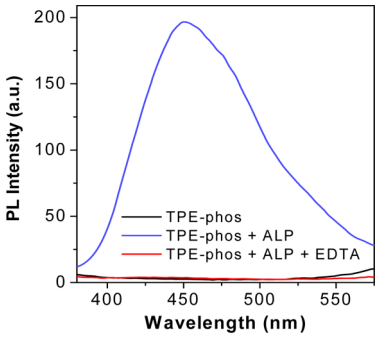

Figure 3. PL spectra of 10 μM TPE-phos alone, and that incubated with ALP (20 nM) in the absence and presence of inhibitor (EDTA) (0.5 mM) in Tris buffer (10 mM, pH 8.0). λ_{ex} = 312 nm; λ_{em} = 450 nm.

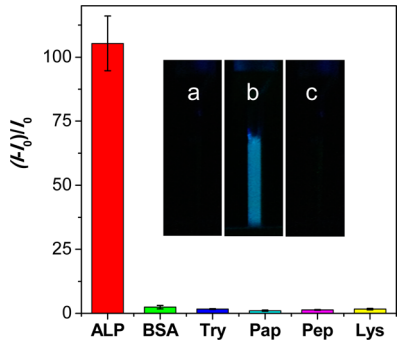

Figure 5. Bar graph of $(I - I_0)/I_0$ of TPE-phos incubated with different proteins in Tris buffer (10 nM, pH 8.0), where I_0 and I are the PL intensities of 10 μM TPE-phos alone and that incubated with 20 nM proteins, respectively. λ_{ex} = 312 nm; λ_{em} = 450 nm. The inset shows the photographs of TPE-phos (a) before and after incubation with (b) ALP and (c) BSA in Tris buffer, respectively, taking under UV lamp illumination.

99%) of Tris buffer were also examined (Figure 4). It is observed that with increasing fraction of Tris buffer, the emission intensity of TPE-2OH (I) stays relatively unchanged until the point of 90% Tris, and dramatically increased to maximum at 99% Tris. These results reveal the existence of aggregates upon hydrolysis of the probe, which contributes to the light-up mechanism.

The selectivity of the probe was then evaluated by comparing its fluorescence response in the presence of different proteins. TPE-phos (10 μM) solution is incubated with ALP and a group of proteins, including bovine serum albumin (BSA), trypsin (try), papain (pap), pepsin (pep), and lysozyme (lys) at the same molar concentration of 20 nM. It is found from Figure 5 that ALP can induce over 100-times brighter fluorescence than other proteins which can be easily distinguished by naked eyes. The exceptionally high selectivity not only elucidates the existence of specific interaction between TPE-phos and ALP, but also signifies that the probe can be used for discrimination of ALP among interference substances.

To demonstrate the quantification of ALP using TPE-phos, the fluorescence intensity of the probe incubated with ALP at different concentrations is monitored. TPE-phos (10 μM) and ALP with concentration of 0−60 nM are incubated for 20 min and their fluorescence intensity at emission maxima was recorded by microplate reader. As seen from Figure 6A, fluorescence intensity increases as the concentration of ALP increases, which is an indication of higher extent of enzymatic reaction and the production of more TPE-2OH. A perfect linear range was plotted in the inset of Figure 6A. Based on the 3 times standard deviation (3σ) method, the limit of detection was evaluated to be 11.4 pM or 0.2 U L^{-1}, which is much lower than or comparable with that obtained by previous reports.[11,14,26,28] A linear response range of 3−526 U L^{-1} was observed.

To test the ability of the probe for detection in biological samples, the assay was performed in 2% fetal bovine serum (FBS). Figure 6B shows the fluorescence response of the probe after incubation with ALP at different concentrations in 2% FBS. The fluorescence progressively lights up as the concentration of ALP increases and it saturates at a lower concentration than that performed in the buffer due to the presence of interfering substances in the serum. Nonetheless, a linear response range up to the ALP concentration of 10 nM or 175 UL^{-1} is obtained which covers the normal level of serum ALP of 30−135 UL^{-1}. These results indicate that the probe can be used for quantification of ALP in complex media, which is potentially useful for diagnosis of ALP related diseases.

We further applied the probe for study of ALP activity. To calibrate the fluorescence intensities of completely digested substrate TPE-phos as a function of substrate concentration, the fluorescence intensity of TPE-phos at different concen-

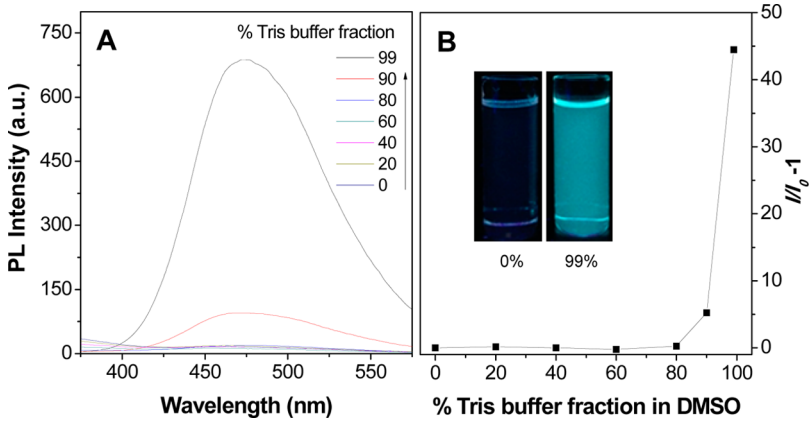

Figure 4. (A) PL spectra of TPE-2OH in different % Tris buffer fractions in DMSO. (B) Plot of I/I_0 − 1 of TPE-2OH in different % Tris buffer fraction in DMSO at 476 nm, where I_0 is the PL intensity of TPE-2OH in 0% Tris buffer. Inset: image of TPE-2OH in 0 and 99% Tris buffer fraction in DMSO taken under UV lamp at 365 nm illumination. ([TPE-2OH] = 10 μM; λ_{ex} = 312 nm.).

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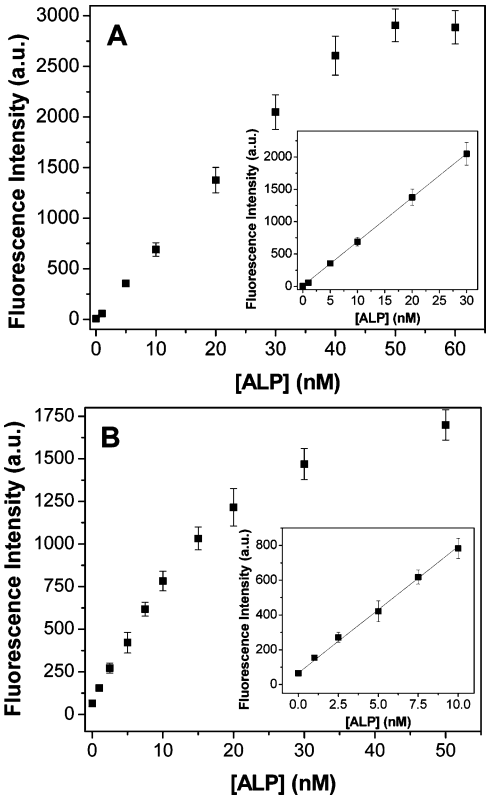

Figure 6. Plots of fluorescence intensity of 10 μM TPE-phos in (A) Tris buffer (10 nM, pH 8.0) or (B) 2% FBS incubated with ALP at different concentrations for 20 min at room temperature. The insets show the linear fitting of the curve at ALP concentrations in ranges of (A) 0–30 nM and (B) 0–10 nM. λ_{ex} = 312 nm; λ_{em} = 450 nm.

trations (2.5–10 μM) was recorded after incubating with excess enzyme for complete hydrolysis (Figure 7A). Subsequently, the fluorescence intensity of TPE-phos at different concentrations (2.5–50 μM) incubated with 20 nM ALP is monitored as a function of time. As shown in Figure 7B, the fluorescence intensities of the substrate increases with time, indicating that the progressive enzymatic reaction takes place. The fluorescence changes rapidly in the early stage and gradually levels off at around 5 min, which suggests that the enzyme cleaves efficiently. The digestion of the probe was also confirmed by LCMS-IT-TOF. As shown from Figure S3 in the Supporting Information, after incubation of the probe with 20 nM ALP, the peak at ~9.5 min disappeared and a new peak appeared at ~13.1 min, which mass matches well with that of the product TPE-2OH. These results indicate that TPE-phos is completely cleaved to TPE-2OH after 20 min incubation with ALP.

The kinetics of the enzymatic hydrolysis reaction is represented by the Michaelis–Menten relations $V_0 = V_{max}[S]/(K_M + [S])$,[29] where V_0 is the initial rate or velocity, [S] is the concentration of substrate, and K_M is the product of k_{cat} and enzyme concentration $[E]_0$. The Michaelis constant K_M is the substrate concentration at which the reaction rate reaches half the maximum, and its inverse is the measure of the affinity of the substrate toward the enzyme. The turnover number k_{cat} is defined as the maximum amount of substrate hydrolyzed per unit enzyme per unit time. In this study, V_0 is expressed as the change of fluorescence intensity per unit time at early stage and is plotted against substrate concentration [S] in Figure 7C. To determine the kinetic parameters, the curve is converted to a

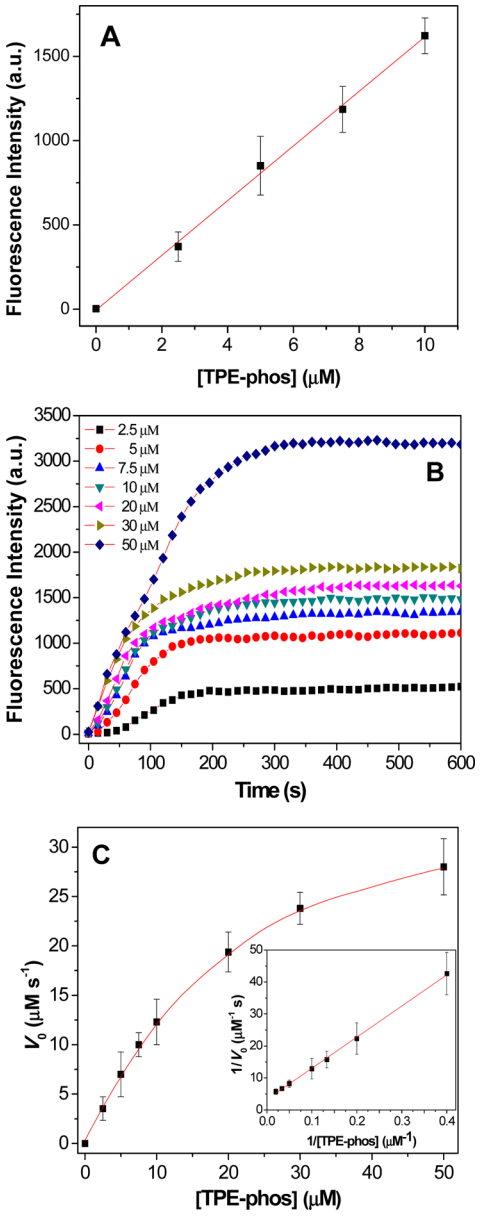

Figure 7. (A) Plot of fluorescence intensity TPE-phos versus TPE-phos concentrations (0–10 μM) after incubation with excess ALP (20 nM) in Tris buffer (10 nM, pH 8.0) for 20 min at room temperature. (B) Time-dependent fluorescence intensity of TPE-phos at different concentrations (2.5–50 μM) incubated with 20 nM ALP at room temperature in Tris buffer (10 nM, pH 8.0). (C) Plot of initial velocity (V_0) against TPE-phos concentration. The inset shows the Lineweaver–Burk plot for the reaction between TPE-phos and ALP. λ_{ex} = 312 nm; λ_{em} = 450 nm.

linear line using Lineweaver–Burk analysis, $1/V_0 = K_M/k_{cat}[E_0][S] + 1/k_{cat}[E_0]$.[29] As shown in the inset of Figure 3B, the plot of $1/V_0$ versus $1/[S]$ fits a straight line. On the basis of the slope and intercept of the fitted line, the k_{cat} and K_M are determined to be 15.1 s^{-1} and 29.3 μM, respectively. The enzymatic efficiency, as measured by k_{cat}/K_M, is then calculated to be 5.1 × 10^5 M^{-1} s^{-1}, which is close to that of a commercial substrate 4-methylumbelliferyl phosphate (4-MUP) from Invitrogen measured under similar conditions (see Figure S4 in the Supporting Information). In addition, TPE-phos can induce a much higher (~3-fold) signal-to-noise ratio than that of 4-MUP because of the extremely low background of the AIE

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probe as witnessed in the insets of Figure 1 and Figure S4A in the Supporting Information.

CONCLUSION

In conclusion, a TPE-based light-up probe for detection of ALP and monitoring ALP activity has been developed. The exceptional selectivity and water solubility endowed by phosphate groups are essential for optimal assay performance. The TPE-phos probe is able to detect ALP in pM range with a linear response range in solution and serum, which opens up the potential opportunity for ALP quantification in clinical practice. The strategy presented in this study can easily be generalized for construction of molecular probes for other enzymes through conjugation of target specific ligands to AIE luminogens.

ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization of intermediates and probe; fluorometric assay of ALP with commercial substrate. This material is available free of charge via the Internet at http://pubs.acs.org/.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: cheliub@nus.edu.sg.
*E-mail: tangbz@ust.hk

Author Contributions

‡Authors J. L. and R. T. K. K. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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