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Real-Time Fluorescence Turn-On Detection of Alkaline Phosphatase Activity with a Novel Perylene Probe

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Abstract: A tetracationic perylene probe (probe **1**) was designed and synthesized. Probe **1** was used for the real-time fluorescence turn-on assay of alkaline phosphatase (ALP) activity and inhibitor screening. Probe **1** monomer fluorescence could be very efficiently quenched by ATP through the formation of an ATP/probe **1** complex. ALP triggered the degradation of ATP, the breakdown of the ATP/probe **1** complex, and the recovery of the probe **1** monomer fluorescence. In the presence of an ALP inhibitor, a decrease in fluorescence recovery was observed.

Keywords: aggregation · alkaline phosphatase · biosensors · fluorescence · perylene probe

Introduction

Alkaline phosphatase (ALP, EC 3.1.3.1) is a hydrolase that is responsible for the removal of phosphate functional groups from many types of biomolecules, such as nucleotides, proteins, and alkaloids.[1] ALP is one of the most commonly assayed enzymes, and it plays important roles in many areas of basic biological/biochemical research and clinical diagnosis. A number of assay methods have been developed in recent years for the detection of ALP activity. For example, gold[2] and silver[3] nanoparticles have been used for the colorimetric detection of ALP activity based on the aggregation-induced changes in solution color. Fluorescence-based ALP activity assays have drawn considerable attention because the required instrument is easily accessible and the sensitivity is usually quite high. Several fluorescence probes, such as quantum dots (QDs),[4] conjugated polyelectrolytes (CPEs),[5] and small molecular probes with aggregation-induced emission (AIE) property[6] have been successfully employed for detection of ALP activity. However, certain drawbacks still exist. For example, the synthesis of conjugated polyelectrolytes and nanoparticles is rather complicated, time consuming, and expensive. Some quantum dots are toxic, and the AIE-based assays yield turn-off output detection signals.[7] Therefore, the development of a simple, fast, sensitive, and inexpensive fluorescence turn-on ALP activity assay and inhibitor screening is highly desirable.

Perylene compounds usually have a high fluorescence quantum yield and photostability,[8] and have been used as active components in optical devices[9] and sensors.[10] However, most perylene compounds have a strong tendency of self-aggregation through π–π stacking interactions, which results in strong fluorescence quenching.[11] In this work, a novel cationic perylene derivative, probe **1**, was designed and synthesized. Probe **1** contains four positively charged quaternary ammonium groups around the perylene core and thus shows considerable water solubility (>50 mM). Its UV/Vis absorption maximum is at 470 nm, and it exhibits bright green fluorescence with a maximum emission at 488 nm. In addition, since it contains four positive charges, strong repulsive electrostatic interactions between probe **1** molecules diminish their tendency of self-aggregation. Figure S1 in the Supporting Information shows that the peak emission intensity of probe **1** increased linearly with increasing probe concentration up to 7 μM. The results indicate that probe **1** very weakly self-aggregates and mainly exists in the monomer form in the 0–7 μM concentration range.

We herein report a simple, fast, inexpensive, and sensitive fluorescent turn-on method for assaying ALP activity in real time using **1** as a fluorescence probe. The rationale for the assay is schematically illustrated in Scheme 1 and explained as follows: (1) Probe **1** is strongly fluorescent in an aqueous buffer solution. Adenosine triphosphate (ATP) is a short linear polyelectrolyte and contains four negative charges. ATP can be converted into adenosine by the enzymatic dephosphorylation of ALP. Adenosine contains no net charge. (2) When ATP is added to the solution of probe **1**, strong attractive electrostatic interactions between them and also hydrophobic interactions between probe **1** and the adenine base of ATP result in probe **1**/ATP complex formation and aggregation-induced quenching. (3) Upon the addition of ALP to the assay solution, ATP is degraded, which leads to

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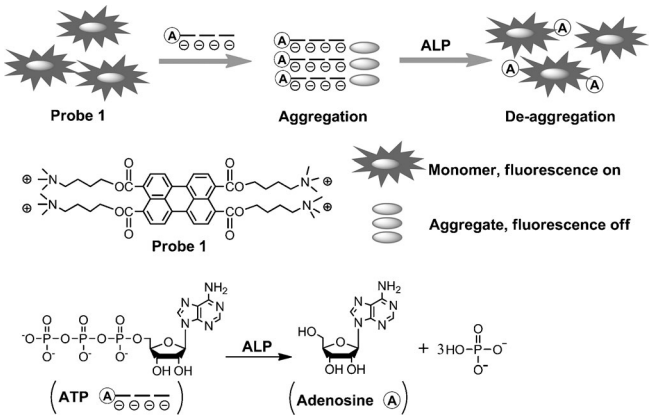


Scheme 1. Schematic illustration of the fluorescence "turn-on" detection of ALP activity with the perylene probe (probe **1**).

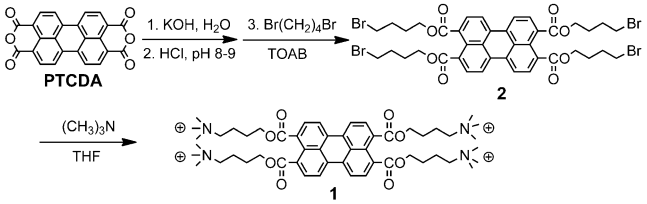
the dissociation of the probe **1**/ATP complex. Probe **1** monomers are released, and a fluorescence turn-on is detected. A convenient fluorometric assay method for ALP activity was therefore established. (4) In the presence of an ALP inhibitor, a decreased in fluorescence recovery would be observed, which could be used for the screening of potential ALP inhibitors.

Results and Discussion

The synthetic route for the perylene probe is shown in Scheme 2. Compound **2** was prepared in 63% yield by direct esterification of the perylene tetracarboxylic acid core with 1,4-dibromobutane at the 3,4,9,10 positions in an oil/water mixture.[12] The crude product was purified by silica column chromatography. Tetraoctylammonium bromide (TOAB) was used as the phase-transfer catalyst. Probe **1** was obtained by ionization of compound **2** with trimethylamine in 95% yield.[13] The correct identity and purity of compounds were assessed by NMR spectroscopy and electrospray ionization mass spectrometry (ESI-MS). In the ^{1}H NMR spectrum of probe **1**, the appearance of a peak with a chemical shift of 3.12 ppm indicates successful ionization of the alkyl chain.

Abstract in Chinese:

摘要：本篇论文设计并合成了一种新型的带正电荷的芘探针（探针 1）。探针 1 可用作碱性磷酸酶活性的荧光实时检测及其抑制剂的筛选。探针 1 能与 ATP 作用形成集聚体，从而导致探针单体荧光的淬灭。碱性磷酸酶能降解 ATP，从而破坏 ATP 和探针的集聚体，进而导致探针 1 单体荧光的恢复。当体系中存在碱性磷酸酶的抑制剂时，荧光的恢复程度降低。



Scheme 2. Synthetic route for probe **1**.

Our results show that the fluorescence intensity of probe **1** (5 μM) decreased gradually when mixed with increasing concentrations of ATP (0–200 μM; Figure 1a). For example, the peak emission intensity of probe **1** decreased nearly 7 times when 200 μM of ATP was introduced. The fluorescence quenching can easily observed by the naked eye, as indicated in the inset in Figure 1a. The corresponding Stern–Volmer plot of the data is shown in Figure 1b. The plot is linear in the low concentration range of ATP. The Stern–Volmer constant (K_{sv}) derived from the linear region of the plot was calculated to be approximately $5.03\times10^4\,M^{-1}$. In addition, the probe **1**-ATP complex, with a calculated binding constant of $4.7\times10^4\,M^{-1}$ (Figure S2 in the Supporting Information), appears to be sufficiently stable under our assay conditions.[14]

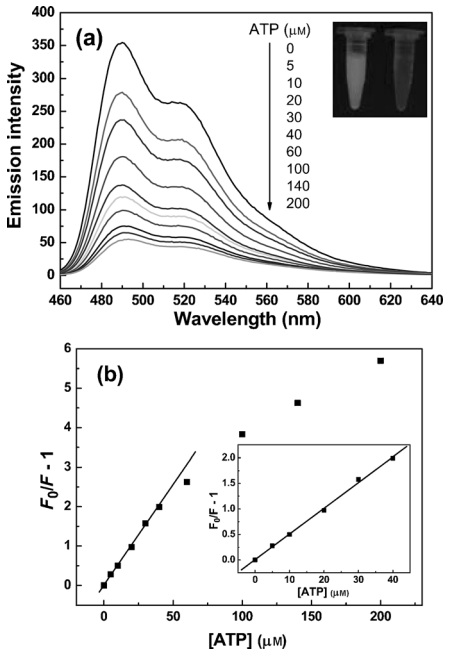


Figure 1. (a) Changes in the emission spectrum of probe **1** (5 μM) upon the addition of ATP at different concentrations (0–200 μM). Inset: photos of solutions of probe **1** in the absence (left) and presence (right) of ATP (200 μM) under illumination by UV light (365 nm). (b) Stern–Volmer plot of fluorescence quenching of probe **1** (5 μM) by different amounts of ATP (0–200 μM). The inset shows the expanded linear region of the curve.

The maximum UV/Vis absorption of probe **1** (Figure S3 in the Supporting Information) was red-shifted by 5 nm to

475 nm along with a noticeable intensity decrease, owing to the formation of ATP-probe **1** aggregate.[15] Dynamic light scattering studies also confirm the aggregation-induced formation of the probe **1**/ATP complex (Figure S4 in the Supporting Information). The results clearly suggest that ATP could induce the aggregation of probe **1**, thereby quenching the fluorescence of monomeric **1**. Figure 2 and Figure S5 in the Supporting Information show that when pyrophosphate

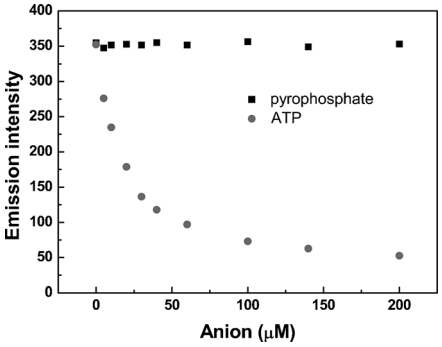


Figure 2. Changes in emission intensity of probe **1** (5 μM) at 488 nm upon addition of pyrophosphate and ATP at different concentrations.

(4 negative charges) was used instead of ATP (4 negative charges), the induced aggregation of probe **1** molecules was hardly observed. Meanwhile, ADP (3 negative charges), AMP (2 negative charges), and adenosine (no charge) show a relatively low effect to induce aggregation and quench the emission of probe **1** (Figure S6 in the Supporting Information). It therefore appears that only combined hydrophobic and attractive electrostatic interactions result in the aggregation of the perylene probe.

ALP can catalyze the removal of the negatively charged 5′-phosphate groups from ATP.[16] Therefore, it was anticipated that the addition of ALP to the solution of probe **1** and ATP would lead to the breakdown of the probe **1**-ATP aggregates because of elimination of the attractive electrostatic interactions. As a result, the fluorescence of the dye would recover. Figure 3 shows the gradual recovery of the fluorescence signal of probe **1** upon the addition of ALP (2.5 U mL^{-1}) to the assay solution. The fluorescence intensity was observed to increase gradually with the reaction time until a plateau was reached after 30 minutes of reaction. The fluorescence recovery of probe **1** could be clearly seen by the naked eye (inset in Figure 3).

The peak emission intensity changes of probe **1** in the presence of ATP (with and without ALP), ADP (adenosine diphosphate), AMP (adenosine monophosphate), and adenosine of the same concentration are shown in Figure S6 in the Supporting Information. The results indicate that most ATP molecules had been converted into adenosine by the enzymatic action of ALP. Furthermore, the blue shift (from 475 back to 470 nm) and intensity increase of the maximum in the UV/Vis absorption of probe **1** after the enzymatic digestion by ALP (Figure S3 in the Supporting Information) also verified this conclusion. We should note that our probe

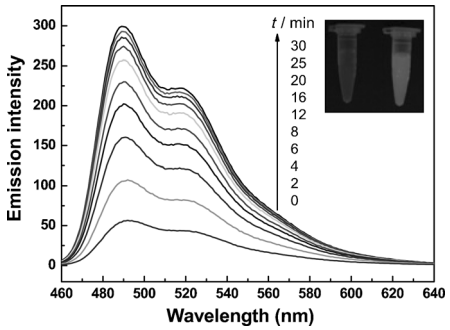


Figure 3. Emission spectra of probe **1** (5 μM) at different reaction times (0–30 min). The assay solution contained 200 μM ATP and 2.5 U mL^{-1} ALP. Inset: photos of solutions of probe **1** and ATP in the absence (left) and presence (right) of ALP (2.5 U mL^{-1}) under illumination by UV light (365 nm). Enzymatic reaction time: 30 min.

utilized only noncovalent interactions with the natural substrate (ATP) for the detection of ALP activity. ALP substrates with similar structures (ADP and AMP) could all induce perylene probe aggregation and be used for the detection of ALP activity, which could be advantageous for practical applications. For example, if the assay mixture contains another enzyme with ATPase activity, the use of ATP for the detection of ALP activity would not be possible. In this case, the use of ADP (or AMP) for the selective detection of ALP activity would be preferred (Figure S7 in the Supporting Information).

The selectivity of the fluorometric ALP assay was also investigated. A number of enzymes such as esterase, trypsin, and exonuclease I (Exo I) were tested. These enzymes can catalyze the hydrolysis of carboxylic esters, proteins, and nucleic acids, respectively. The concentration of each enzyme was kept at 2.5 U mL^{-1}, and each assay solution was incubated for 30 minutes under the same experimental conditions. Figure 4 shows that none of these enzymes had the ability to recover the quenched fluorescence of probe **1** except ALP. The results further confirm that the fluorescence recovery of probe **1** was generated by the ALP-catalyzed dephosphorylation of ATP, which clearly suggests that the current fluorescence turn-on ALP assay is highly selective for ALP. The

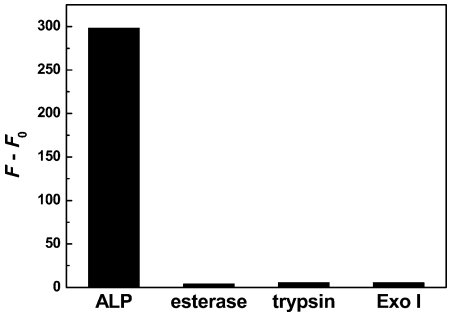


Figure 4. Emission intensity of probe **1** (5 μM) at 488 nm. Assay solutions contained ATP (200 μM) and 2.5 U mL^{-1} ALP, esterase, trypsin, or Exo I, respectively. All solutions were incubated at 25 °C for 30 min. Background emission was subtracted for clarity.

detection of ALP activity was also tested in A549 cell lysates. Figure S8 in the Supporting Information shows that a clear fluorescence recovery of probe **1** was obtained with the addition of ALP to the sample containing diluted cell lysates (1%). The results clearly show that our assay could be used in real-life samples.

Our assay method could be used for monitoring the ALP activity in real time. Continuous probe **1** emission intensity changes at 488 nm monitored in assay solutions containing probe **1** (5 μM), ATP (200 μM), and different amounts of ALP are shown in Figure 5. The results show that the emission intensity of probe **1** increased gradually with the reaction time. The emission intensity increased more quickly and recovered completely at higher ALP concentrations. The linear relationship between the initial reaction rate (V_0) and the enzyme concentration suggests that the dephosphorylation reaction is first order with respect to the ALP concentration, in agreement with previous studies.[17] Our assay method is quite sensitivity compared with previously reported methods;[2b,3,4,5a] the activity of 0.01 U mL^{-1} of ALP could be easily detected (Figure S10 in the Supporting Information). The kinetic parameters of the ALP-catalyzed ATP hydrolysis were determined. The K_m and V_{max} values were estimated to be 18.9 μM and 35.4 μM min^{-1}, respectively (Figure S11 in the Supporting Information).

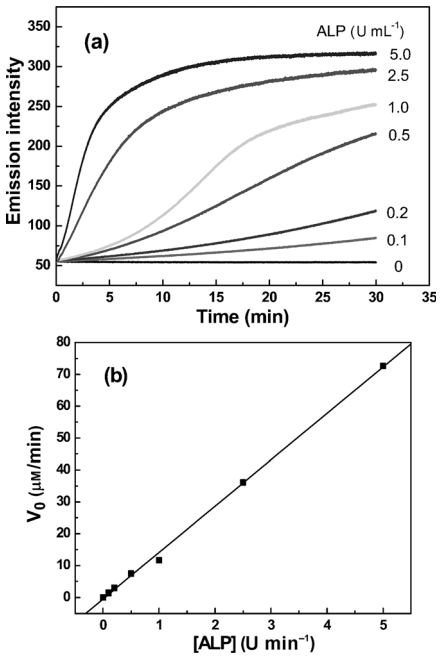


Figure 5. (a) Real-time emission intensity changes of probe **1** (5 μM) at 488 nm. Assay solutions contained 200 μM ATP and different concentrations of ALP. (b) Plot of the initial reaction rate (V_0) as a function of ALP concentration.

The probe **1**/ATP complex described herein could also be used for the screening of potential ALP inhibitors. To demonstrate this possibility, sodium orthovanadate (Na_3VO_4), a well-known inhibitor for ALP,[18] was tested. Figure 6 shows the real-time fluorescence intensity changes of the probe **1**/ATP complex at 488 nm in the presence of ALP (5.0 U mL^{-1}) and different concentrations of Na_3VO_4. The fluorescence recovery of probe **1** decreased significantly with increased inhibitor concentrations, thus indicating that the overall inhibition was more effective at higher inhibitor concentrations. The IC_{50} value of Na_3VO_4 toward ALP was estimated to be 54.7 μM (Figure S12 in the Supporting Information). Similarly, 4,4′-diaminodiphenyl, another ALP inhibitor, was also tested. The fluorescence recovery of probe **1** decreased significantly with increasing 4,4′-diaminodiphenyl concentration, and the corresponding IC_{50} value was estimated to be 343 μM (Figure S13 in the Supporting Information). In addition, *p*-nitrophenyl phosphate was used instead of ATP (Figure S14 in the Supporting Information), and the inhibition effect of 4,4′-diaminodiphenyl was tested. The corresponding IC_{50} value was calculated to be 280 μM (Figure S15 in the Supporting Information). The results clearly demonstrate that our assay could not only be used for the real-time monitoring of ALP activity but also for the screening of potential ALP inhibitors.

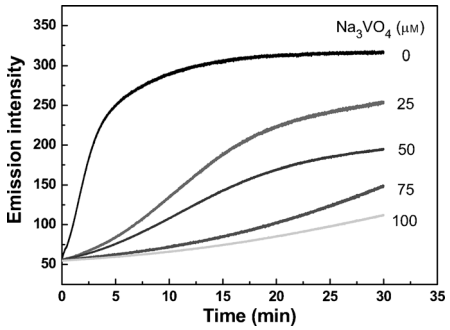


Figure 6. Real-time emission intensity changes of probe **1** (5 μM) at 488 nm. Assay solutions contained 200 μM ATP, 5.0 U mL^{-1} ALP, and the indicated concentrations of Na_3VO_4.

Conclusions

In summary, a facile real-time fluorescence turn-on assay for ALP activity has been developed. Probe **1** is highly soluble in water and self-aggregates negligibly. ATP could induce the aggregation of probe **1** monomers and, consequently, quenches its fluorescence. Upon the addition of ALP, the fluorescence of probe **1** recovered due to the enzymatic dephosphorylation of ALP. The assay is advantageous due to several important features. First, it is based on the fluorescence "turn-on" mode, which could reduce considerably the likelihood of false-positive signals associated with "turn-off" assays. Second, it offers a convenient "mix-and-detect" approach for the rapid and sensitive detection of ALP activity and inhibition. Third, the fluorescence intensity changes can be monitored in real-time using a common spectrophotometer. Forth, probe **1** can be easily prepared, and the substrate (ATP) is commercial available; thus, the assay is fairly cost-effective.

Experimental Section

General Experimental Details

Materials: Alkaline phosphatase (from calf intestine) and Exo I were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Esterase and trypsin were purchased from Sigma–Aldrich (St. Louis, MO, USA). ATP, ADP, AMP, and adenosine were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). 4,4′-diaminodiphenyl and *p*-nitrophenyl phosphate were purchased from TCI (Shanghai, China). 3,4,9,10-perylenetetracarboxylic dianhydride (PTCDA) was purchased from Acros Organics. Tetraoctylammonium bromide (TOAB) and 1,4-dibromobutane were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and used without further purification. All stock and buffer solutions were prepared using water purified with a Milli-Q A10 filtration system (Millipore, Billerica, MA, USA).

Measurements: ^{1}H NMR spectra were obtained with a Bruker AVANCE 600 (600 MHz) Fourier transform NMR spectrometer with chemical shifts reported in parts per million (ppm) relative to tetramethylsilane. Splitting patterns are reported as s (singlet), d (doublet), t (triplet), and multiplet (m). UV/Vis absorption spectra were obtained using a Cary 50 Bio Spectrophotometer (Varian Inc., CA, USA) equipped with a xenon flash lamp. Emission spectra were recorded using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc., USA) with an excitation wavelength of 442 nm and corrected against PMT response. Excitation and emission slit widths were both 2 nm at 5 μM probe **1** concentration. Quartz cuvettes with 10 mm path length and 2 mm window width were used for UV/Vis and emission measurements. Unless otherwise specified, all spectra were taken at 25 °C in 5 mM Tris-HAc buffer at pH 8.2.

Probe Synthesis

Synthesis of 3,4,9,10-tetra-(4-bromobutyloxy-carbonyl)-perylene (2): Perylene-3,4,9,10-tetracarboxylic dianhydride (PTCDA) (784 mg, 2.0 mmol), KOH (1.0 g, 17.7 mmol), and 30 mL deionized water were added into a 100 mL flask and stirred at 70 °C for 30 min. The solution was filtered and its pH value was adjusted to 8–9 with 1 M HCl. Subsequently, tetraoctylammonium bromide (TOAB) (400 mg, 0.7 mmol) was added. The mixture was stirred vigorously for 10 min, and 1,4-dibromobutane (4.3 g, 20.0 mmol) was added. The solution was refluxed with vigorous stirring for 2 h. The aqueous solution became clear and colorless, and on top of it a layer of red oil formed. Next, $CHCl_3$ (30 mL) was added to the reaction mixture. The organic phase was washed three times with 15% aqueous NaCl (30 mL) and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using a mixture of CH_2Cl_2/petroleum ether (1:1) as eluent. After drying under vacuum, compound **2** (1.2 g, 63%) was obtained as an orange solid. ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 8.37$ (d, 4H), 8.07 (d, 4H), 4.36–4.40 (t, 8H), 3.48–3.52 (t, 8H), 2.03–2.10 (m, 8H), 1.95–2.02 ppm (m, 8H); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 167.9$, 132.4, 129.8, 129.5, 128.3, 128.2, 120.9, 64.0, 32.7, 28.8, 26.6 ppm.

Synthesis of 3,4,9,10-tetra-(4-trimethylammoniobutyloxy-carbonyl)-perylene (1): Compound 2 (484 mg, 0.5 mmol) was dissolved in THF (50 mL) in a 100 mL flask. An excess amount of trimethylamine (5 mL, 30% aqueous solution) was added. The solution was refluxed for 3 days. During this period, water was added at several intervals (total of 15 mL). The organic solvent was evaporated under reduced pressure, and the aqueous solution was washed with $CHCl_3$ (30 mL) three times. After solvent evaporation and drying under vacuum, probe 1 (0.57 g, 95%) was obtained as a red solid. ^{1}H NMR (600 MHz, D_2O): $\delta = 7.62$–7.69 (m, 8H), 4.39–4.43 (t, 8H), 3.38–3.42 (t, 8H), 3.12 (s, 36H), 1.87–1.93 (m, 8H), 1.93–1.99 ppm (m, 8H); ^{13}C NMR (150 MHz, D_2O): $\delta = 169.1$, 130.9, 129.7, 127.7, 126.6, 126.5, 120.7, 65.6, 65.0, 52.6, 24.5, 19.1 ppm; ESI-MS: m/z 221.34 [M^{4+}/4] (see Figure S21 in the Supporting Information).

Fluorescence Quenching

ATP (0–200 μM) was added to the solution of probe **1** (5 μM) in Tris-HAc buffer (5 mM, pH 8.2). The solutions were thoroughly mixed for subsequent fluorescence and absorption measurements. The K_{sv} value was calculated using the Stern–Volmer equation: $F_0/F = 1 + K_{sv}[Q]$, in which F_0 and F refer to the fluorescence intensity in the absence and presence of the quencher, respectively, and [Q] refers to the concentration of the quencher in μM.[18]

Binding Constant Calculation

Probe **1** (5 μM) and different amounts of ATP were mixed in a Tris-HAc buffer solution and the corresponding UV/Vis spectra were recorded. The binding constant was calculated according to the following equation: $[ATP]/(\varepsilon_A - \varepsilon_F) = [ATP]/(\varepsilon_B - \varepsilon_F) + 1/K_a(\varepsilon_B - \varepsilon_F)$, where ε_A, ε_B, and ε_F are A_{obs}/[probe], the extinction coefficient of probe **1** in the fully bound form, and the extinction coefficient of free probe **1**, respectively.[14] Next, $[ATP]/(\varepsilon_A - \varepsilon_F)$ was plotted versus ATP concentration. The binding constant (K_a) was determined as the ratio of the slope to the intercept (Figure S2 in the Supporting Information).

Fluorescence Recovery

ALP (2.5 U mL^{-1}) was added to the solution of probe **1** (5 μM) and ATP (200 μM) in Tris-HAc buffer (5 mM, pH 8.2). The resulting solution was incubated at 25 °C for a certain period of time (0–30 min), and the emission spectrum was measured.

Assay Selectivity

ALP, esterase, trypsin, or Exo I (2.5 U mL^{-1} each) were added to a solution of probe **1** (5 μM) and ATP (200 μM) in Tris-HAc buffer (5 mM, pH 8.2). The solutions were incubated at 25 °C for 30 min, and the emission spectra were recorded.

Kinetic Assays

Different amounts of ALP (0, 0.01, 0.02, 0.1, 0.2, 0.5, 1.0, 2.5, and 5.0 U mL^{-1}, respectively) were added to a solution of probe **1** (5 μM) and ATP (200 μM) in Tris-HAc buffer (5 mM, pH 8.2), and emission intensity changes at 488 nm were monitored in real time. The concentration of ATP in the assay solution at a certain reaction time was estimated from the Stern–Volmer curve. The initial reaction rate (V_0) from the linear portion of the kinetic curve was then calculated (Figure S9 in the Supporting Information).

ALP Inhibition

Different amounts of the inhibitors (0, 25, 50, 75, and 100 μM for Na_3VO_4; 0, 100, 200, 500, and 1000 μM for 4,4′-diaminodiphenyl) were added to the solution of probe **1** (5 μM), ATP (200 μM), and ALP (5.0 U mL$^-$) in Tris-HAc buffer (5 mM, pH 8.2), and real-time emission spectral changes of probe **1** at 488 nm were monitored. The inhibition efficiency was defined by the following equation:

$$(IE) = [F_{(no\ inhibitor)} - F_{(inhibitor)}]/[F_{(no\ inhibitor)} - F_0]$$

in which $F_{(inhibitor)}$ and $F_{(no\ inhibitor)}$ refer to the emission intensity of probe **1** in the presence or absence of inhibitors, and F_0 refers to background emission of probe **1**.[7b] The IC_{50} value refers to the inhibitor concentration required for 50% inhibition of the enzymatic activity.

Acknowledgements

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