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Monoaromatic Fluorescence Probe for Sensitive Alkaline Phosphatase Analysis by Regulating Intramolecular Hydrogen Bonding

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Enzymatic reactions have been widely applied to the design of various sensing systems. However, the detection sensitivity of partial systems is still low, possibly attributed to the large molecular size of the substrate. In this study, we propose a strategy toward sensitive enzyme analysis with monoaromatic substrate. That is, salicyl phosphate ester (SPE) as a fluorimetric probe is presented for evaluating the enzymatic activity of alkaline phosphatase (ALP). The sensing mechanism is that ALP catalyzes the dephosphorylation cleavage of SPE and generates salicylic acid with strong fluorescence by modulating intramolecular hydrogen bonding. Due to the specific phosphorylation-dephosphorylation chemistry, the SPE based probe shows high specificity toward ALP over other possible interferents. The SPE probe provides a limit of detection of 0.005 U L^{-1}. In addition, the practical application of the SPE probe is supported by ALP determination in human serum.

Keywords: monoaromatic probe, fluorescence, intramolecular hydrogen bonding, alkaline phosphatase, serum sample

Introduction

Enzymatic reactions are critical to various biological processes, and most reactions still work *in vitro*.[1-3] Such a character makes enzymatic reactions very popular in the design of versatile detection systems toward diverse targets, including enzymes, proteins, deoxyribonucleic acids (DNAs), anions and even metal ions.[4-11] In general, the targets can be the enzyme, substrate or inhibitor. For example, Dai *et al.*[12] proposed 1,8-naphthalimide derivatives for fluorescent detection of cytochrome P450 1A enzyme. Mao *et al.*[13] reported the glucose (substrate) sensing in human body by glucose oxidase modified magnetic nanomaterials. As is known, organophosphorus compounds can inhibit the catalytic activities of many enzymes, and thus numbers of optical and/or electrochemical detection approaches toward organophosphorus pesticides have been developed based on enzyme-involved systems with an indirect design.[14] For most enzymatic systems, the specificities are generally high enough due to the special reaction pathway or steric conformation.[15] However, the sensitivities of partial enzymatic systems are still poor, which may hinder the rapid and effective identification of targets. Therefore, the exploration of a simple enzymatic system with high sensitivity is appealing.

As is known, enzymatic reactions usually undergo a binding-to-product process.[16,17] That is, the enzyme-substrate binding intermediate is first formed, which subsequently catalyzes the activation of substrate and generation of product. In consideration of the pathway, the efficiency of enzymatic reaction can be improved by structural matching.[18-20] For example, the catalytic efficiency of the same substrate can be improved by changing substation positions.[21] It has been reported that the kinetics and productivity of some chemical reactions can be promoted by reducing the molecular sizes of reactants,[22] thus it is feasible to enhance the efficiency of enzymatic reactions by involving small substrate molecules, which weakens the spatial hindrance.

Phosphorylation is an important reaction that regulates the biological functions, and the resulting phosphorylated products can be transformed into free substrates by phosphatase-mediated dephosphorylation.[23,24] Detection of phosphatase activity is essential to screen the physiological status, and thus attracts growing attention in the development of selective and sensitive probes.[25-28] Among them, organic probes with good selectivity have been explored.[7,29-31]

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In some cases, the designed organic probes show poor sensitivity, partially due to the large molecular size and spatial hindrance. Herein, a monoaromatic probe (salicyl phosphate ester, SPE) was proposed for the evaluation of the activity of alkaline phosphatase (ALP) by regulating the intermolecular hydrogen bonding. The schematic ALP-mediated catalyzed SPE hydrolysis was shown in Figure 1. The ALP-induced dephosphorylation cleavages SPE and produces salicylic acid (SA), and the chemical reaction was unambiguously confirmed by UV-Visible absorption spectra, fluorescence spectra and mass spectrometry. By integrating invisible fluorescence of SPE and salicylic acid formation via specific ALP-SPE reaction, turn-on ALP detection with high selectivity was achieved. With the reduction of molecular size of enzymatic substrate, the proposed SPE probe sensitively responded toward ALP with a limit of detection (LOD) of 0.005 U L^{-1} (signal-to-noise ratio (S/N) = 3). The response over other possible interferents was also studied. In addition, the practical application of SPE probe for ALP test in serum samples analysis was verified with high accuracy.

Experimental

Chemicals and materials

Salicyl phosphate ester (SPE) was obtained from Macklin Chemical by custom synthesis (Shanghai, China). Alkaline phosphatase (ALP) was provided by Hunan Zhixiangweilai Biotechnology (Changsha, China). Inorganic salts, including potassium chloride, magnesium chloride, calcium chloride, sodium chloride, sodium bicarbonate, and sodium sulfate (Na_2SO_4) were obtained from Fuchen Chemical (Tianjin, China). Amino acids and peptide, such as L-aspartic acid (Asp), L-alanine (Ala), glycine (Gly), L-serine (Ser), and glutathione (GSH, reduced form) were purchased from Sinopharm Chemical Reagent (Shanghai, China). Glucose (Glu) and 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) were obtained from Beijing chemical (Beijing, China). Bovine serum albumin (BSA) and lysozyme (Lys) were purchased from Merck (New Jersey, USA). Other chemicals were obtained from Tokyo Chemical Industry (TCI, Shanghai, China). All chemicals were directly used in different assays without purification. Ultrapure water (18.2 MΩ cm) was supplied by a Millipore system. The pH buffer solutions were prepared based on the standard protocols.

Apparatus

UV-Vis absorption spectra were characterized with UV3600 spectrophotometer (Shimadzu, Kyoto, Japan). The absorbance was recorded in the range of 190 to 400 nm. Fluorescence excitation and emission spectra were obtained using F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). For fluorescence measurements, a 600 V potential was chosen for all assays. Electrospray ionization mass spectroscopy (ESI-MS) was recorded with negative ESI mode on a Xevo G2 Q-Tof mass spectrometer (Waters, Massachusetts, USA). The negative model ESI-MS was performed with 6 L min^{-1} dry gas, 200 ºC dry temperature, and 3300 V capillary voltage.

Sensitivity and specificity evaluation of SPE probe

SPE stoke solution (500 μM) was first prepared, other SPE solutions with different concentrations were obtained through serial dilution. The enzymatic activity evaluation assays were performed by monitoring the fluorescence signal variation of SPE in the presence of ALP. In brief, SPE probe was first diluted with 10 mM HEPES buffer solution (pH 7.4) to make a final concentration of 5 μM. Then, ALP with different activities (0 to 5.0 U L^{-1}) was introduced into the solution. To ensure a sufficient reaction, the mixed solution was incubated at 37 °C for 40 min. Finally, the fluorescence emission spectra of resulted solution were acquired through fluorescence spectrophotometer with 294 nm excitation. The specificity of SPE probe toward ALP was examined by changing ALP into different possible interferents, including metal ions, amino acids, and small molecules. Briefly, SPE probe was first diluted with 10 mM HEPES buffer solution (pH 7.4) to make a final concentration of 5 μM. Then, 1 mM

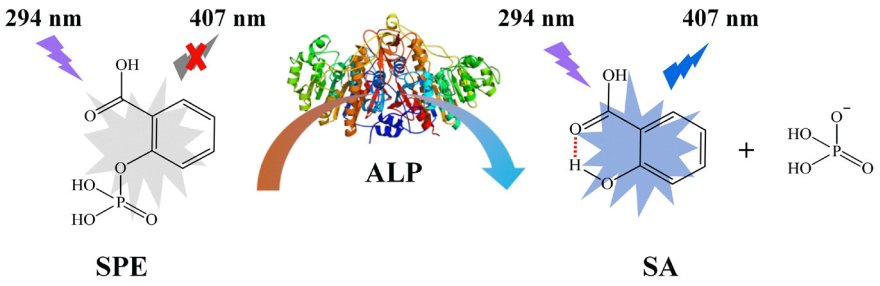

Figure 1. Schematic illustration of SPE-based turn-on detection of ALP by regulating intramolecular hydrogen bonding.

interferents were introduced into the solution in the absence or presence of ALP (1.0 U L^{-1}). After 40 min incubation at 37 °C, the fluorescence emission spectra of resulted solution were acquired through fluorescence spectrophotometer with 294 nm excitation.

ALP analysis in serum samples

The ALP analysis in human serum sample was investigated to illustrate the practicability of the proposed SPE probe. The serum samples were obtained from healthy postulants without take of any drugs in two months before test. The cells in serum sample were first removed by centrifugation (10000 rpm, 15 min). The resulting supernatant was collected and then re-dispersed in buffer. Before the test, any large aggregate was eliminated through membrane filtration (0.22 μm). Finally, 5 μM SPE was introduced into the re-dispersed and diluted serum sample. The fluorescence spectra were recorded after 40 min reaction under 37 °C. The use of human samples in this study was approved by the Beijing University of Chemical Technology under protocol No. KY230424005.

Results and Discussion

ALP-mediated fluorescence enhancement of SPE probe

The fluorescence emission of SA with intramolecular hydrogen bonding emitted strong blue fluorescence under UV light irradiation. As shown in Figure 2a, single emissive component was observed. The maxima excitation wavelength was located at 294 nm, while the maxima emission wavelength was around 407 nm. Accordingly, the maximum absorbance appeared around 300 nm (Figure 2b), which is consistent with the excitation wavelength. However, the maximum absorbance of SPE shifted to 275 nm (Figure 2c), which is attributed to the intrinsic absorption of benzene ring. In comparison to SPE, one intramolecular hydrogen bonding exists in SA. As mentioned in other works, intramolecular hydrogen bonding reduces the energy of conjugation and induces red-shift of UV-Vis absorption and fluorescence emission.[32] Thus, the major reason of the blue shifted absorption wavelength in SPE is possibly attributed to the phosphorylation-induced break of intramolecular hydrogen bonding. Without intramolecular hydrogen bonding, SPE only showed weak emission under irradiation (Figure 2d). It is well known that phosphatase can catalyze the breakage of phosphate ester and liberates free hydroxyl group, which makes dephosphorylation of phosphorylated products. To understand whether the dephosphorylation of SPE can be achieved upon adding phosphatase, the optical signal variation of SPE was investigated by the introduction of ALP. As shown in Figure 2e, the fluorescence of SPE was dramatically enhanced after adding ALP. Meanwhile, a new absorption peak of SPE solution around 300 nm belonging to SA appeared with the addition of ALP (Figure 2f). The simultaneously changed fluorescence and UV absorbance indicated the ALP-induced dephosphorylation of SPE,[33]

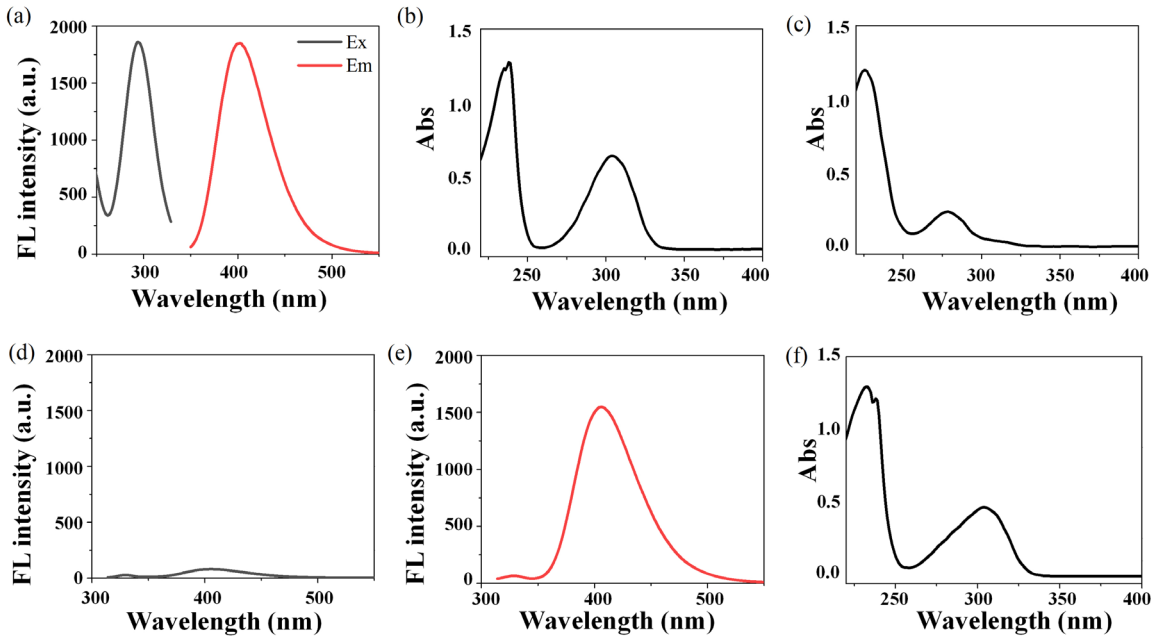

Figure 2. Optical characterization of SPE in the absence or the presence of ALP. (a) The steady-state fluorescence spectra (Ex for excitation and Em for emission) of SA. (b) The UV-Vis absorption spectra of SA. (c) The UV-Vis absorption spectra of SPE. The steady-state emission spectra of SPE in the absence (d) and the presence (e) of ALP. (f) The UV-Vis absorption spectra of SPE after adding ALP.

suggesting the possibility of ALP activity screening with SPE.

To demonstrate the formation of SA, ESI-MS characterizations of SPE before and after the addition of ALP were conducted. As shown in Figure 3, the *m/z* peak at 216.9954 belongs to SPE $[M - H]^-$ (calcd. for $C_7H_6O_6P$: 216.9907) and the *m/z* peak at 137.0256 belongs to SA $[M - H]^-$ (calcd. for $C_7H_5O_3$: 137.0244) appeared. It was seen that no signal around 137.0256 could be observed in Figure 3a, ruling out the self-hydrolysis of SPE without addition of ALP. After the introduction of ALP, the intensity of *m/z* peak at 216.9899 dramatically decreased, indicating the consumption of SPE. Meanwhile, a new and strong *m/z* peak around 137.0234 appeared. This peak can be attributed to generated SA, proving the dephosphorylation of SPE and regeneration of SA. The optical variations and MS results demonstrated that ALP-induced fluorescence enhancement is assigned to the ALP-mediated dephosphorylation of SPE and the regeneration of SA. Therefore, fluorimetric screening of ALP activity by using SPE as probe is feasible.

Establishment of SPE-based ALP detection platform

As referred above, ALP can induce the dephosphorylation of SPE and promote the regeneration of fluorescent SA, thus it is possible to indicate the ALP activity by fluorescence signal variation. Herein, we aimed to explore a SPE-based fluorescence probe for the monitoring of ALP activity. To realize sensitive ALP detection, few parameters that affect the enzymatic activity were investigated first. It is reported that ALP possesses pH dependent activity,[21] and thus the effect of solution pH should be considered. In order to reveal the pH effect on ALP activity, the steady-state fluorescence emission spectra of SPE solution (final concentration: 5 μM) without and with the addition of ALP under different pH conditions were recorded. After 40 min reaction under 37 ºC, the intensity ratio of I/I_0 at 407 nm was determined, which can be used for ALP activity evaluation. In a word, the large I/I_0 ratio value suggests the high activity. As shown in Figure 4a, the I/I_0 ratio increased quickly from pH 6.5 to 8.0, while it deceased with further increment of pH. However, the fluorescence of SPE solution was visible when the solution pH decreased to 6.5, possibly due to the acid-induced hydrolysis of SPE. As reported in few works, phosphorylated compounds are instable in strongly acidic environment. High I/I_0 ratio was observed under pH 7.5 to 8.5, and the maximum appeared with pH 8.0. Therefore, all following assays were conducted at pH 8.0.

It should be noticed that ALP usually works in biological conditions, and thus it might be sensitive to the reaction temperature. The influence of reaction temperature on ALP sensing was also studied. As a protein-based enzyme, high temperature may lead to the inactivation of ALP. To avoid the inactivation, temperature below 50 ºC was considered. Briefly, the fluorescence signals of SPE solution without and with the addition of ALP under the 25-50 ºC temperature range were recorded. As manifested in Figure 4b, the I/I_0 ratio showed gradual increment as the temperature increased from 25 to 37 ºC. However, further increase of temperature led to the decrease of I/I_0 ratio. Interestingly, all I/I_0 ratio values were larger than 10 even with 50 ºC reaction temperature, indicating ALP is highly active. On the basis of the result, subsequent experiments were performed at 37 ºC.

The conformational variation during enzyme-substrate binding decides the efficiency of enzymatic reactions,[16] and thus the reaction time is important for catalytic process and efficiency. To ensure the complete reaction, the time-dependent fluorescence variation of SPE probe in the presence of ALP was monitored. As displayed in Figure 4c, once the ALP was added, the I/I_0 value gradually increased and reached a flatform after 30 min. However, long reaction time (> 60 min) led to a slight decrease in fluorescence. To obtain high sensitivity, reaction time of 40 min was chosen.

Many practical applications of proposed probes toward ALP analysis are performed in serum. However, the complicated matrix of human serum samples, especially high salt concentration may inhibit the accurate detection.

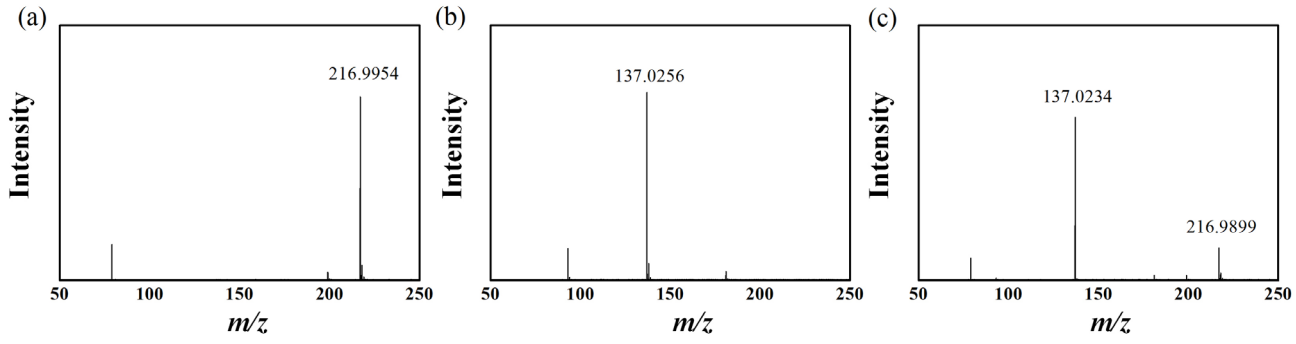

Figure 3. ESI-MS spectra of SPE (a), SA (b) and SPE + ALP (c) with negative-ion model.

Therefore, the salt tolerance of SPE probe should be tested. The salt effect on ALP-induced fluorescence response was also studied. It was seen that the addition of NaCl did not cause visible variation in fluorescence enhancement even with with high concentration (0.1 M, Figure 4d). The maintained I/I_0 values under wide NaCl concentration range suggest the high stability of SPE probe toward ALP analysis in complicated environments, even in biological media.

Sensitivity and selectivity evaluation of ALP activity detection

Under the optimal condition, the ALP-induced fluorescence variation was applied for the evaluation of the enzymatic activity. It was seen that the strong emission of SPE solution appeared after the introduction of ALP. According to Figure 5a, the fluorescence emission intensity of SPE solution showed a gradual increase with increased ALP concentration. As manifested in Figure 5b, the fluorescence ratio (I/I_0) displayed a rapid increment with the ALP concentration from 0 to 3.0 U L^{-1}, and then reached a flatform. The relationship between I/I_0 and the ALP concentration could be well-described with the following equation: $I/I_0 = 0.73 + K[Q]$, where K is the enzymatic reaction constant, and [Q] is the ALP concentration. On the basis of linear regression, the constant K was calculated to be 8.29×10^9 M^{-1}, which is much higher than that of many reported fluorimetric sensors. Interestingly, the linear regression coefficient (R^2) was determined to be 0.996, indicating the good linearity of the established equation in the ALP concentration from 0.005 to 2.5 U L^{-1}. The LOD of SPE probe towards ALP was calculated to be 0.005 U L^{-1} (S/N = 3), which is lower than that of some previously reported organic ALP probes (Table 1).[32-43] In general, substrates with small molecular size can easily form a binding intermediate and facilitate the reaction. As Jiang *et al.*[44] referred, the glycogen (a complex branched glucose polymer) enzymatic degradation efficiency is related to its molecular-size, and smaller glycogen particles show faster degradation rate *per* monomer unit. In comparison to polycyclic molecule substrate, SPE with small size would be easily hydrolyzed. In addition, the formed SA product with intramolecular hydrogen-bonding increases its stability, which may benefit the formation of SA and promote the hydrolysis of SPE probe. As a result, SPE probe displayed high sensitivity toward ALP over other polycyclic substrates. As is known, the reproducibility is an important evaluation index for sensors. In order to test the reproducibility of SPE probe, parallel response experiments were conducted with the same reaction conditions. Here, the 1.0 U L^{-1} ALP-induced fluorescence response of SPE probe was repeatedly measured 20 times. As shown in Figure 6, the I/I_0 values in twenty repeated measurements did not show visible difference. The relative standard deviation (RSD) was 1.2%, suggesting the high stability of the proposed SPE probe.

The specificity toward target is also important to judge the analytical performances of the proposed sensors. To

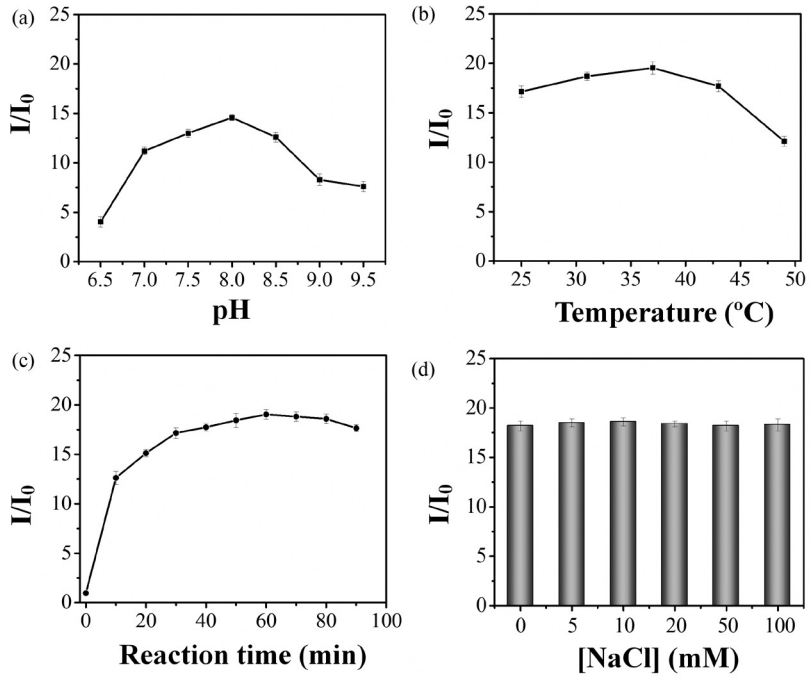

Figure 4. Optimization of ALP sensing with SPE probe. Fluorescence intensity ratio of SPE upon adding 2.0 U L^{-1} ALP *versus* solution pH (a), incubation temperature (b), reaction time (c), and salt concentration (d), respectively. the excitation wavelength was set to be 294 nm, and the emission intensity was collected at 407 nm.

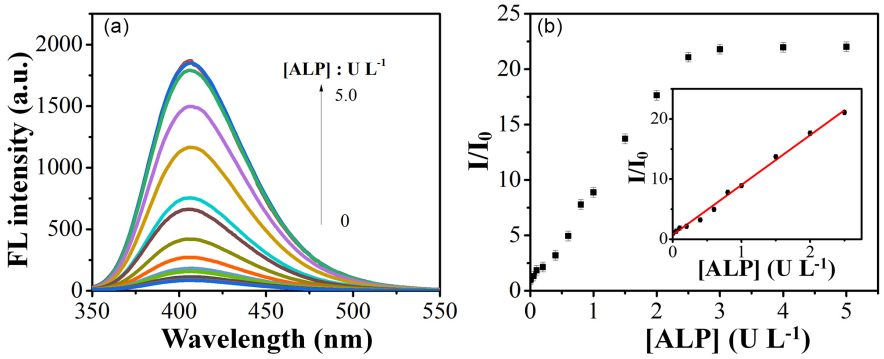

Figure 5. Fluorescence response of SPE probe toward ALP with various concentrations. (a) The fluorescence emission spectra of SPE solution with the addition of various concentrations of ALP. (b) Plots of I/I_0 value *versus* ALP concentration in corresponding concentration range. The linear response expression was displayed in the inset image.

Table 1. Comparison of this work with some proposed fluorimetric ALP sensing probes

No.	Probe name	Fluorescence strategy	LOD / (U L^{-1})	Linear range / (U L^{-1})	Reference
1	QX-P	turn-on	17	50-1000	34
2	HBTP-mito	ratiometric	0.072	0-60	35
3	SWJT-3	ratiometric	0.87	0-150	36
4	AA2P+DAN	ratiometric	0.08	0.1-60	37
5	HP	ratiometric	3.98	0-250	38
6	Ratio-ALP	ratiometric	0.25	0-200	39
7	AA2P+OPD	ratiometric	0.06	0.5-22	40
8	N3-CR-PO4	turn-on	0.01	0.01-1.0	41
9	TPE-peptide	turn-on	1.2	1.0-1000	42
10	LET-3	turn-on	50	50-600	43
11	DHBA	turn-on	0.005	0.005-2.5	this study

LOD: limit of detection.

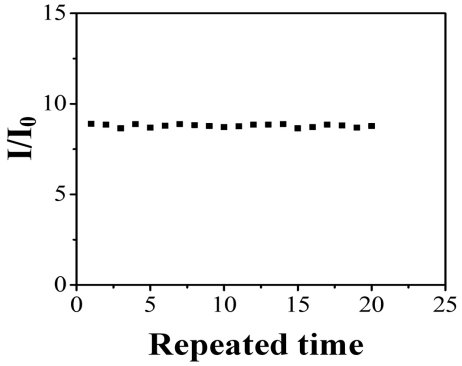

Figure 6. Twenty repeated measurements of I/I_0 value of SPE probe in the presence of 1.0 U L^{-1} ALP.

figure out whether the ALP induced fluorescence increment is specific, the fluorescence responses of SPE probe towards various possible interferents were investigated. In this work, some metal ions, small molecules, amino acids and proteins were chosen as the interferents, including K^+, Ca^{2+} Mg^{2+}, Ala, Asp, Arg, GSH, Ser, Gly, Glu, BSA, and Lys. The concentrations of metal ions, small molecules and amino acids were 1 mM. The concentrations of BSA and Lys were 200 μM. According to the marks of ALP product, 1.0 U L^{-1} is equivalent to 1 nM. This means the concentrations of interferents were at least 200-folds higher than that of ALP. Amazingly, there was no comparable fluorescence response was observed upon adding these interferents (Figure 7a), indicating the ALP-induced fluorescence increment is specific. On the other hand, the tolerance of SPE probe was also investigated in the presence of these interferents. It was seen that the ALP-induced fluorescence response was not affected by the co-existence of 1000-folds possible interferents (Figure 7b). Therefore, the good selectivity, as well as the satisfying tolerance, demonstrates the probe SPE probe could be used for specific ALP activity screening. Taken together, the good sensitivity and specificity imply the potential application of SPE probe for sensitive and selective ALP determination in biological samples.

ALP analysis in real samples

The satisfying analytical performances mentioned above implies that SPE probe is capable to ALP analysis in biological media. Here, the practical application of SPE probe

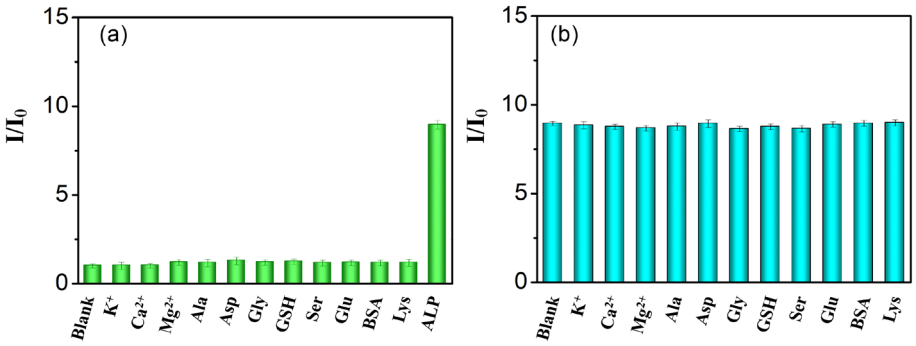

Figure 7. I/I_0 values induced by various possible interferents in the absence (a) and the presence (b) of ALP.

was investigated by ALP analysis in human serum samples. As reported, the ALP content in human serum is usually higher than 30 U L^{-1}.[45] To ensure that the concentration of ALP falls in the linear region, dilution of human serum samples was conducted before test. The existence of ALP was confirmed by the increased fluorescence of SPE solution upon introducing serum sample. The ALP content in serum was determined to be 71.52 ± 0.23 U L^{-1} (Table 2), which is consistent with previous reports. Interestingly, the RSD values were less than 2.5%, indicating the high accuracy of ALP sensing. In addition, the recoveries ranged from 97.8 to 101.3%, which further proved the accuracy of SPE-based ALP sensing platform. Taken together, the proposed SPE probe is adaptable of ALP analyzing in both aqueous and biological media.

Conclusions

In conclusion, we have proposed a monoaromatic substrate toward enzymatic reaction with high sensitivity. In a demo case, non-fluorescent and monoaromatic SPE is applied for fluorimetric ALP sensing, because ALP catalyzes its dephosphorylation and forms fluorescent SA product. The favorable specificity is donated by the intrinsic enzymatic chemistry. By using the SPE probe, rapid ALP detection with a LOD of 0.005 U L^{-1} has been realized. Moreover, the potential application of SPE in real ALP analysis is validated in serum substrate. This work proves the possibility of fluorimetric probe design by modulating intramolecular hydrogen bonding, and also provides a new strategy for sensitive enzyme sensing with monoaromatic substrates. Therefore, through modulating intramolecular hydrogen bonding, it is feasible to design functional and sensitive organic probes based on monoaromatic substrates for various applications in analytical chemistry or biomedical diagnosis.

Author Contributions

X. Z. conceived the experiments; X. Z. and Y. C. carried out the experiments; Y. C. contributed to the data analysis; X. Z. contributed to the writing of this manuscript. All the authors have reviewed the manuscript and agreed to its publication.

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Table 2. SPE-mediated ALP analysis in human serum sample

Sample	Detected / (U L^{-1})	Spiked ALP / (U L^{-1})	Found / (U L^{-1})	Recovery / %	RSD (n = 3) / %
Serum	71.52 ± 0.23	2.00	73.49	98.5	2.4
		3.00	74.49	99.1	1.5
		4.00	75.93	101.3	1.3
		5.00	76.41	97.8	1.6

ALP: alkaline phosphatase; RSD: relative standard deviation.

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