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A Fluorescence Turn-On Bio-sensing Strategy for Sensitive and Rapid Determination of Alkaline Phosphatase in Human Blood Based on the Photo-physical Phenomenon of Aggregation Induced Emission

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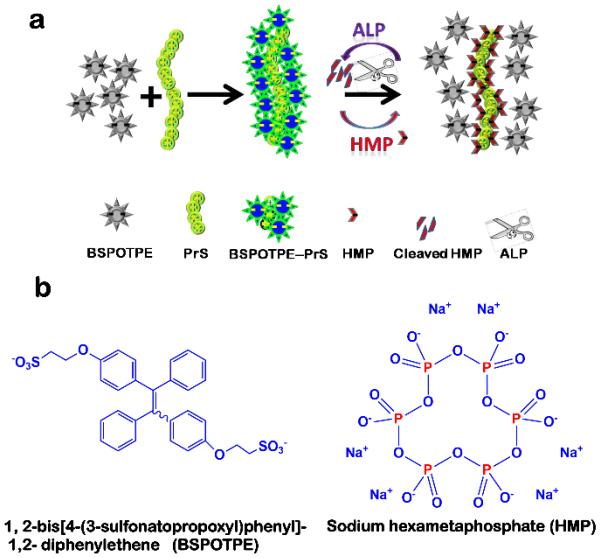
Abstract
Alkaline phosphatase (ALP), membrane bound glycoprotein, catalyses the hydrolysis of phosphoester linkage from carbohydrates, proteins or nucleic acids in basic conditions. ALP is widely distributed in human body. Therefore, the enzyme is also used for biological applications, such as, gene expression and antibody conjugate immunoassays. ALP levels remain 40-160 U/L in healthy adults and, ~500 U/L in children and pregnant women. Several abnormalities, such as, anaemia chronic nephritis, hepatobiliary diseases, and hypothyroidism are caused if the level of ALP is disturbed in the body. Thus, ALP detection is highly significant for diagnostic and pathological assays. In this regard, there exist a variety of assays for ALP detection. However, these methods exhibit major drawback of complex and expensive design, and also low sensitivity. Therefore, it is still very important to develop some inexpensive, sensitive and simple ALP detection approaches. In this work, using the phenomenon of aggregation induced emission (AIE), a rapid, easy, sensitive and highly selective fluorescence "Turn-On" probe is developed to monitor ALP activity. The sensing units of the probing system are made up of an AIE probe dye, di-anionic 1,2-Bis[4-(3-sulfonatopropoxyl) phenyl]-1,2-diphenylethene salt (BSPOTPE) and poly-cationic protamine sulphate (PrS) that electro-statically interact with each other to make a highly fluorescent BSPOTPE-PrS supra-molecular complex. Subsequently, the fluorescence of the complex is efficiently quenched by hexa-anionic hexa-meta-phosphate (HMP). A rapid recovery in the fluorescence, referred as, fluorescence "Turn-On" is obtained by adding ALP in the basic buffer conditions. The sensing platform has been thoroughly investigated using various spectroscopy-based techniques. The sensing probe exhibits limit of detection of 28.7 (±3) µU/mL and linear concentration range of 0–36 mU/mL. The potential application has also been demonstrated in human serum samples.

Keywords: Aggregation Induced Emission, Fluorescence, Alkaline phosphatase, Enzyme, Bio-sensor, Blood.

Introduction
Alkaline phosphatase (ALP), membrane bound glycoprotein, catalyses the hydrolysis of phosphoester linkage from carbohydrates, proteins or nucleic acids in basic conditions. ALP is widely distributed in various organs; more in liver and bones, and less in kidneys, placenta and intestine. In a healthy adult, the ALP levels remain 40-160 U/L, however, ALP amount reaches up to 500U/L in children and pregnant women. Abnormal levels of ALP cause severe diseases, such as, anaemia chronic nephritis, hepatobiliary diseases, and hypothyroidism 1. The enzyme is also used for biological applications such as gene expression and antibody conjugate immunoassays. Thus, ALP detection is highly significant for diagnostic and pathological assays. In this regard, a variety of assays have been developed for the detection of serum ALP levels 2,3. However, these methods exhibit drawbacks, therefore, it is still very important to develop some innovative, convenient, inexpensive, sensitive and simple ALP detection approaches. In this work, we have exploited the unique phenomenon of aggregation induced emission (AIE) to design a rapid, easy, sensitive and highly selective fluorescence "Turn-On" probing system for monitoring ALP activity. The sensing units of the probing system are made up of an AIE probe dye, di-anionic 1,2-Bis[4-(3-sulfonatopropoxyl) phenyl]-1,2-diphenylethene salt (BSPOTPE) and polycationic protamine sulphate (PrS) that electrostatically interact with each other to make a highly fluorescent BSPOTPE-PrS supra-molecular complex. Subsequently, the fluorescence of the complex is efficiently quenched by sodium hexa-anionic hexametaphospahte (HMP). A rapid recovery in the fluorescence referred as fluorescence "Turn-On" is obtained by adding ALP in the basic buffer conditions (Scheme 1).



Scheme 1. a) Schematic illustration of ALP detection strategy using BSPOTPE-PrS supramolecular assembly. b) Chemical structures of BSPOTPE and Hexametaphosphate.

Experimental Section

All chemical and salts were purchased from Sigma Aldrich. BSPOTPE concentration was calculated using molar absorptivity of ~16400 M^{-1} cm^{-1} (at 312) nm. All experiments were performed in 10 mM tris-buffer, pH 9 at 25 oC. All enzymatic reactions were performed following an incubation of 10 minutes at 37 ºC. The ground-state absorption and steady-state fluorescence measurements were performed using Jasco UV-Vis spectrophotometer (model V-650) and fluorimeter (model FP-8500) respectively at room temperature 25 °C using 1cm quartz cuvette. The 5% human serum was prepared in 10 mM tris-HCl, pH 9. The excited-state decay kinetics were collected using a spectrometer (IBH Inc, UK), based on time correlated single photon counting (TCSPC) principle. For the detection of ALP activity, BSPOTPE (1.2 µM) and PrS (264 nM) was mixed. Finally, 1.1 µM of HMP was used to quench the fluorescence. The fluorescence recovery was achieved by ALP addition to the reaction mixture for 10 minutes. The steady-state fluorescence intensity was measured at λex 320 nm, slit width Ex/Em 5/5. The ALP activity was also measured in 5% human serum. Similarly, selectivity experiments were performed.

Results and Discussion

At the outset, we have first introduced the key photophysical factors of supramolecular assembly between BSPOTPE-PrS. The steady-state emission spectra of anionic TPE derivative (BSPOTPE) were recorded in the aqueous buffer conditions (Tris-HCl, 10 mM, pH 9) at various concentrations of cationic polyelectrolyte PrS. An evident, a gradual and strong fluorescence enhancement is observed at 470 nm with gradual addition of PrS that approaches saturation at a PrS concentration of ~300 nM. Overall, a ~270 fold enhancement in emission intensity of BSPOTPE is observed in the presence of ~300 nM concentration of PrS in pure aqueous solution. Further, with an aim to construct a sensor platform for ALP, we have employed HMP, a multiple

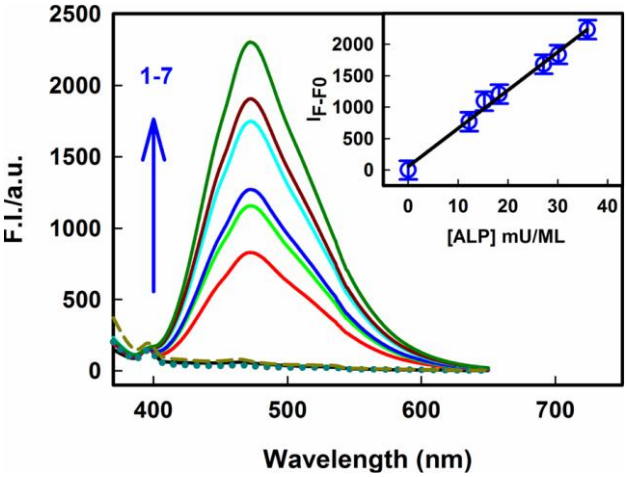


Fig. 1. Fluorescence recovery in 10 mM Tris buffer (pH 9) upon adding various concentrations (mU/mL) of ALP at 37 ◦C for 10 min (1) 0 (2) 12 (3) 15 (4) 18 (5) 27 (6) 30 (7) 35. The steady-state emission spectra of colloidal silica solution, known as Ludox, (dotted, dark cyan line) and BSPOTPE in the presence of Ludox (dashed, dark yellow line) have been also shown which show insignificant emission contribution. Inset: Plot of changes in emission intensity at 470 nm versus ALP concentration, where, the blue circles represent individual data points around the linearly fitted solid black line. The error bar represents the standard deviation of three successive measurements.

Negative charge bearing substrate for ALP, which may bind to cationic PrS to cause disaggregation of BSPOTPE from BSPOTPE-PrS complex. To test this hypothesis, emission response of BSPOTPE-PrS aggregates at various concentrations of HMP has been recorded. It is visible that the emission of BSPOTPE-PrS is drastically quenched with a gradual increment of HMP concentration in the solution, and saturation is observed beyond ~1.5 µM of HMP concentration which mimics the condition of free BSPOTPE in the solution. The catalytic cleavage of HMP in the presence of enzyme ALP may facilitate disaggregation of HMP-PrS complex to make PrS available for interacting electrostatically with BSPOTPE molecules to re-establish BSPOTPE-PrS complex. To validate this hypothesis experimentally, increasing concentrations of enzyme ALP (0-36 mU/ml) were added to an aqueous buffered solution (10 mM Tris buffer, pH 9) of BSPOTPE-PrS-HMP system, incubated for 10 minutes at 37 oC, and steady-state emission spectra were measured (Figure 1). Further, the response of ALP was found to be linear in the concentration range of 0-36 mU/ml, and the regression equation for the fitted data is I(F-F0) = 60.7 [ALP/mU/mL] +56.3 with a regression coefficient (R2) of 0.992. The limit of detection was determined to be 28.7 (±3) µU/mL using the formula 3σ/s, where, σ denotes standard deviation from the mean value for 10 blank measurements, and s is the slope of the straight line fit.

The selectivity of the BSPOTPE-PrS-HMP system towards ALP detection has been determined using different types of enzymes and proteins such as bovine serum albumin, lysozyme, haemoglobin (Hb), acetylcholinesterase (AChE), glucose oxidase (GOx), pepsin, trypsin, and horse radish peroxidise (HRP), and the results clearly indicate that HMP acts as a selective substrate for ALP (Figure 2). Finally, the potential feasibility of the current sensing method in detecting ALP in real samples has been demonstrated in human serum samples.

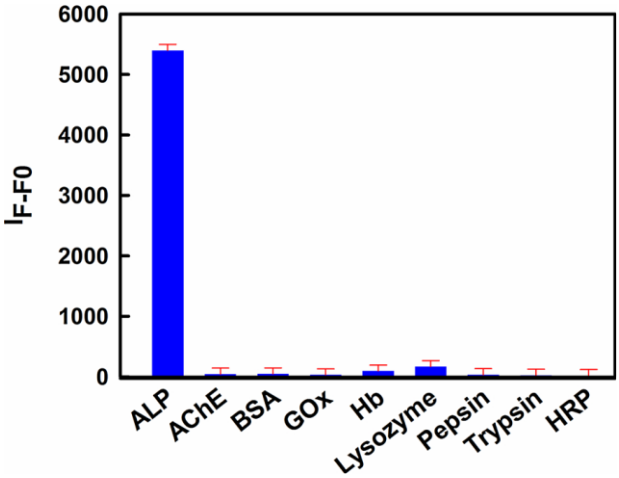


Figure 2. Specificity of the BSPOTPE-PrS-HMP sensing system for ALP detection over other proteins and enzymes at a concentration of 40 mU/mL. The error bar denotes the standard deviation of three replicate readings.

Conclusion

A sensitive, fast, and simple AIE based fluorescence "Turn-On" probing system has been developed to detect ALP, a crucial biomarker enzyme present in human blood. BSPOTPE, an anionic derivative of TPE, interacts with a cationic polyelectrolyte protamine to assemble into a fluorescent BSPOTPE-PrS complex. The AIE effect of BSPOTPE-PrS complex is hampered by adding a quencher of fluorescence, HMP, an anionic molecule carrying six negative charges which outweighs doubly negatively charged BSPOTPE from the PrS surface based on electrostatic considerations. The fluorescence of the disintegrated BSPOTPE-PrS complex is again regenerated via enzymatic breakdown of phosphoester linkages of HMP by adding ALP in the basic buffer solution which serves as a "Turn-On" detection method for ALP. Current system is found to be highly selective towards ALP. The current ALP detection system is able to monitor as low as 28.7 µU/mL of ALP within a linear range of 0–36 mU/mL in 10 min at 37 ◦C with excellent selectivity. Furthermore, the applicability of the probing system in detecting ALP in real samples is also demonstrated by spiking human serum with ALP at various concentrations. Thus, the BSPOTPE-PrS based fluorescence "Turn-On" sensor is a convenient, sensitive, selective and rapid approach to measure ALP in the diagnostic and clinical practices. Thus, the BSPOTPE-PrS based fluorescence "Turn-On" sensor is a convenient, sensitive, selective and rapid approach to measure ALP in 100 % aqueous medium with broad pH range of operation that is expected to significantly assist in ALP related biochemical research.

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