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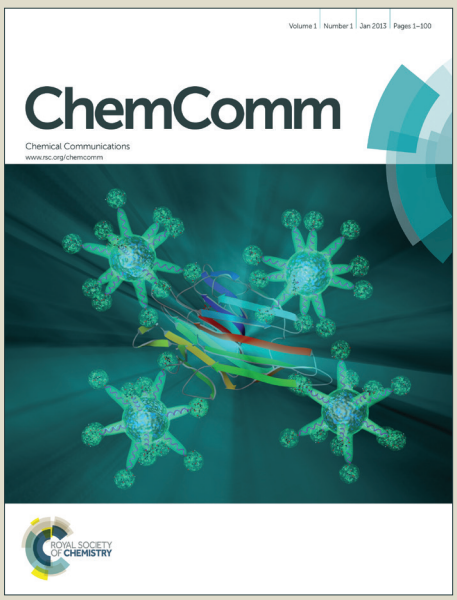

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Enzyme-activatable Probe with a Self-immolative Linker for Rapid and Sensitive Alkaline Phosphatase Detection and Cell Imaging through Cascade Reaction

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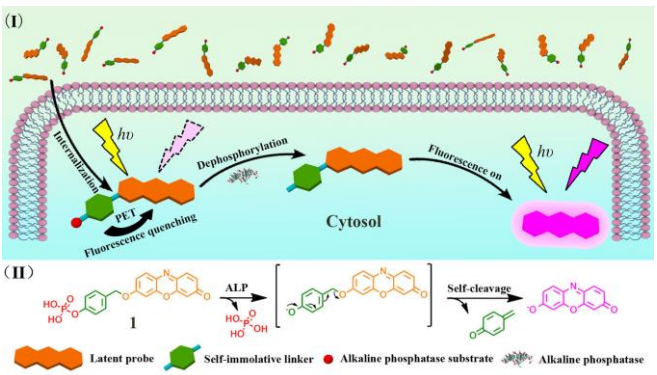
Hongmei Zhang,[a] Chenglong Xu,[a] Jie Liu,[a] Xiaohong Li,[a] Lin Guo*[b] and Xinming Li*[a]

We report the design and synthesis of a novel probe (1) for ALP assay by incorporating a self-immolative linker between phosphate moiety and resorufin. Because of its good biocompatibility and rapid cell internalization, this probe also exhibited great potential for real-time monitoring of endogenous phosphatase activity in living cells.

Alkaline phosphatase (ALP) is a group of isoenzyme widespread in mammalian tissues and responsible for the hydrolysis of phosphate ester groups from various protein and non-protein substrates.[1] The elevated levels of its activities in parts of the human body are well documented to be associated with many diseases in numerous cells and tissues, including bone diseases, liver dysfunctions, breast and prostatic cancers and diabetes.[2] Therefore, development of rapid, sensitive and biocompatible probes for ALP detection is highly desirable to meet the requirements of studying the dynamic ALP activities in different biological systems.[3]

In the last decade, the studies of molecular probe, which can respond to the presence of a specific analyte and rapidly and selectively turn on its fluorescence, have received great attention.[4] Compared to conventional fluorescent probes (fluorescence always-on), analyte activatable fluorescence probe exhibited a distinct advantage (i.e., high signal-to-background ratio), which led to enhanced contrast and sensitivity of the probe during the process of analyte detection and cellular imaging.[5] Based on this strategy, numerous systems with fluorescence turn-on properties have been developed for ALP detections with the applications of fluorogens with aggregation-induced emission characteristics,[6] complexes of cationic polymers and other negative components,[7] or fluorescent nanoparticles incorporated with ALP substrates.[8, 9]

Despite many achievements from the above-mentioned successful works, the system with the potentials for both real-time ALP assay and cell imaging was still rare.[10] So it is highly desirable to develop a simple, fast, and low cost probe suitable for ALP study. In this work, we designed and synthesized a novel probe (**1**) through simple conjugation of a phosphate moiety (the substrate of ALP) to a pro-fluorophore via a reactive *p*-hydroxybenzyl group (a self-immolative linker). We chose resorufin as the pro-fluorophore because of its good solubility, nontoxicity, and unique colorimetric and fluorogenic signal properties.[11] Its obvious colour change and fluorescence turn-on emission from resorufin at 585 nm can be easily observed by naked-eye and read-out in instruments upon interaction with an analyte. On the other hand, the incorporation of a self-immolative linker between the phosphate moiety and resorufin can greatly reduce steric hindrance and enhance the accessibility of probe **1** towards ALP for dephosphorylation reaction.[12]

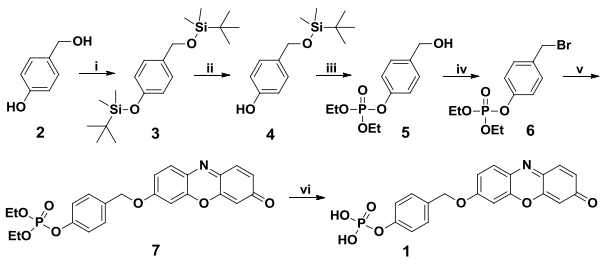


Scheme 1 Illustration of the fluorescence turn-on process of probe **1** triggered by ALP for enzymatic detection and cell imaging.

As illustrated in Scheme 1, when the phosphate moiety on probe **1** was subject to dephosphorylation reaction catalysed by ALP, a self-immolative reaction occurred via 1, 6-elimination of the *p*-hydroxybenzylether derivative and released resorufin properly, leading to the colour change of the solution from orange to purple and the generation of strong fluorescence emission at 585 nm upon photo irradiation. The quinone methide residue generated concomitantly could be quenched by the bulk medium (e.g., H_2O), and/or other nucleophiles near the site of reaction in cells.[13] Based on this strategy,

this probe exhibited the capabilities for rapid and sensitive ALP detection in solution through both naked-eye observation and fluorescence detection, and the detection limit can be as low as 1.09 U/L. Furthermore, because of its good biocompatibility and rapid cell internalization, this probe also exhibited the potentials for real-time monitoring of endogenous phosphatase activity in living cells.

Scheme 2 shows the synthetic route for the preparation of latent probe **1**. The synthesis work started with the protection of dihydroxyl groups of *p*-hydroxybenzyl alcohol (**2**) with *tert*-butyldimethylsilane chloride. The resulted compound **3** was then subject to selective deprotection of the aryl silyl ether group with the application of cesium carbonate, leading to the formation of compound **4**. After phosphorylation reaction with diethyl chlorophosphate, **4** was converted to compound **5**, which further underwent bromination reaction to form **6** by using carbon tetrabromide and triphenylphosphine as reagents. Alkylation of resorufin with **6** with the presence of potassium carbonate afforded compound **7**. Followed by subsequent de-ethylation reaction by using trimethyliodosilane, **7** was converted to probe **1** in yield of 40%. The chemical structures of these compounds were characterized by both NMR spectroscopy and electrospray ionization mass spectrometry (ESI-MS), both of which gave satisfactory analysis data corresponding to their expected molecular structures (Fig. S1-S7).



Scheme 2 The synthetic routes for the preparation of fluorogenic probe **1**.

With the purpose of investigating the sensitivity of probe **1** towards ALP, we firstly dissolved probe **1** in 10 mM phosphate buffer to make a clear solution (10 μM, pH=7.4) in light orange colour. As shown in Fig. 1, probe **1** exhibited a broad UV absorption peak at 484 nm, a shoulder peak at around 400 nm (Fig. 1A), and a very weak fluorescence emission band around 585 nm (quantum yield $\Phi \approx 0.0023$), which were consistent with the non-emissive character of probe **1**, due to the quenching effect of 7-hydroxy substitution on resorufin via photo-induced electron transfer (PET) (Fig. 1B).[14] However, after the addition of ALP (1.0 U/mL), the solution changed the colour from light orange to pink rapidly, which could be easily discriminated by naked-eye under visible light (Fig. S8). In addition, from UV-visible and fluorescence spectroscopy analysis, we found that the solution showed a strong UV absorption peak centred at 574 nm (Fig. 1A), and remarkable fluorescence emission peak at 585 nm (Fig. 1B), indicating the generation of resorufin from the induced self-immolative reactions of probe **1** catalysed by ALP.

In order to confirm that the colour change and fluorescence enhancement at 585 nm is due to the dephosphorylation reaction of probe **1** triggered by ALP, we used ^{31}P NMR spectroscopy to study the transformation of latent probe **1** upon the treatment with ALP. Probe

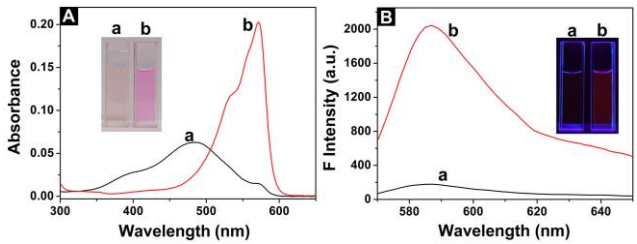


Fig. 1 (A) The UV absorption spectra and (B) fluorescence emission spectra of **1** (10 μM, pH=7.4, λ_{ex}=550 nm) before (a, black) and after (b, red) the addition of ALP (1.0 U/mL). Inset: The optical images of the corresponding solutions of **1** (10 μM, pH=7.4) in the absence (a, left) or presence (b, right) of ALP (1.0 U/mL) under (A) visible and (B) UV light irradiation (365 nm).

1 was firstly dissolved in 10 mM Tris-HCl buffer (pH=7.4), which contained additional 10% v/v deuterium (D_2O) to yield probe **1** solution with concentration at 2.5 mM. From the ^{31}P NMR spectra shown in Fig. 2A, we observed the presence of a strong resonance peak at -0.22 ppm, which corresponded to the phosphate ester group attached to probe **1** via the linkage of *p*-hydroxybenzylether group. With the addition of ALP (1.4 U/mL), this resonance peak at -0.22 ppm decreased gradually with time, and a new peak appeared around 2.42 ppm and increased concomitantly till 300 min, which was ascribed to the phosphoric acid released from probe **1** by ALP (Fig. S9). This result indicated that probe **1** can work as an efficient substrate of ALP to undergo dephosphorylation reaction. Furthermore, the hydrolysis of probe **1** by ALP at different time-scale was monitored by fluorimetry. As shown in Fig. 2B, the solution of probe **1** emitted very weak fluorescence without the presence of ALP. However, a remarkable fluorescence emission peak appeared at 585 nm after the addition of ALP (1.0 U/mL). Its intensity increased gradually with time, and reached a plateau in 20 min, indicating the complete transformation of probe **1** to resorufin. The extended dephosphorylation times during the ^{31}P NMR spectroscopy study were due to much higher concentration of probe **1** and the presence of deuterium oxide, which seems to affect the enzymatic efficiency of ALP.

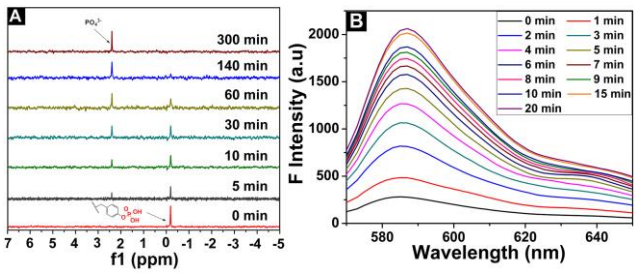


Fig. 2 (A) ^{31}P NMR spectra of **1** (2.5 mM) treated by ALP (1.4 U/mL) at different time scales; (B) time evolution of fluorescence emission spectra (λ_{ex}=550 nm) of the mixture solution containing 10 μM of **1** and 1.0 U/mL ALP.

Furthermore, we conducted dynamic studies to investigate the ALP-catalysed hydrolysis of probe **1** as a function of time using different amounts of ALP. Fig. 3A shows the increase of fluorescence intensity at 585 nm of probe **1** solution with the presence of different amounts of ALP (0-1.0 U/mL). It is obvious that the fluorescence

intensity increased notably and sharply at higher concentration of ALP, which means that increasing the enzyme concentration gave rise to a higher cleavage reaction rate and less time was required to complete the hydrolysis process of probe **1**. Moreover, on the basis of the plot of the relative fluorescence intensities at 585 nm of **1** solution versus the concentrations of ALP ranging from 0.01 to 1.0 U/mL, we calculated the detection limit of probe **1** towards ALP, which can be as low as 1.09 U/L under these conditions (Fig. S10), which is much lower than that of other reported probes.[9, 15] Encouraged by these results, we further determined the kinetic parameters of ALP-catalysed hydrolysis of **1** by incubating ALP (1.0 U/mL) with different concentration of **1** (0.5, 1, 2, 5, 10 and 20 μM, respectively). As shown in Fig. 3B, the fluorescence intensities of the solutions were highly dependent on the concentrations of probe **1**, and the increase of fluorescence intensity was rapid in the early progress and it tended to level off after 60 min, indicating the progressive enzymatic reaction and generation of resorufin from probe **1** by ALP. By directly fitting the data to Lineweaver-Burk plot (Fig. S11), we got the K_M and k_{cat} values of the enzymatic hydrolysis reaction of probe **1**, which were estimated to be 15.38 μM and 0.26 s^{-1}, respectively. Enzymatic efficiency of probe **1**, as estimated by a k_{cat}/K_M = 1.7 x 10^4 M^{-1} s^{-1}, was more than that of the commercially available ALP fluorogenic substrates, 4-MUP (k_{cat}/K_M = 7.7 x 10^3 $M^{-1}s^{-1}$), indicating the high affinity between probe **1** and ALP and efficiency of dephosphorylation reaction catalysed by ALP.

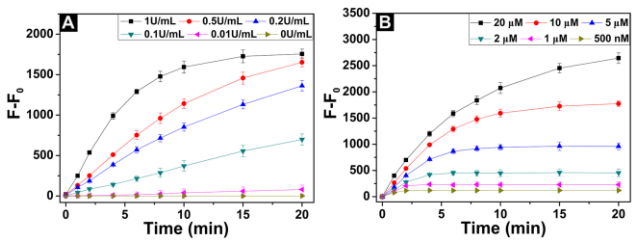


Fig. 3 Time evolution of the fluorescence intensities of the mixture solution at 585 nm, containing (A) 10 μM of probe **1** and different concentrations of ALP (0, 0.01, 0.1, 0.2, 0.5 and 1.0 U/mL); and (B) varied amount of probe **1** (0.5, 1.0, 2.0, 5.0, 10 and 20 μM) and 1.0 U/mL of ALP.

In order to confirm that the introduction of self-immolative linker between resorufin and phosphate group can lead to high enzymatic efficiency of probe **1** to ALP, we synthesized another probe without the presence of *p*-hydroxybenzyl group, namely resorufin-7-O-phosphate, which consisted of only phosphate and resorufin (Scheme S1 and Fig. S12-S14). As shown in Fig. S15-S18, resorufin-7-O-phosphate can exhibited proper responses to the presence of ALP by changing its solution color from light orange to pink and increasing the fluorescence emission at 585 with time, but at a much reduced rate (K_M=24.6 μM, k_{cat}=0.160 s^{-1} and k_{cat}/K_M=6.58×10^3 $M^{-1}s^{-1}$) in comparison with probe **1**, indicating the advantageous effects of incorporating a self-immolative linker between the phosphate group and the resorufin residue to reduce steric hindrance and enhance the accessibility of probe **1** towards ALP for dephosphorylation reaction.

To examine the selectivity of probe **1** towards phosphatase, we conducted control experiment by treating **1** with other nonspecific proteins, such as human serum albumin (HSA), bovine serum albumin (BSA), pepsin, trypsin, esterase, lysozyme and ALP under same conditions. As shown in Fig. 4A, the solution mixed with ALP displayed an intense fluorescence signals at 585 nm, which was about 45-fold higher than that of other proteins, indicating the high selectivity of **1** towards phosphatase. In addition, since the hydrolysis activity of ALP can be greatly inhibited with the presence of inhibitors, we examined the possibility of our probe for inhibitor screening assays. We selected two common ALP inhibitors, namely sodium orthovanadate and levamisole, for this study. As shown in Fig. 4B, the enhancement of the fluorescence intensity of probe **1** was inhibited in a dose-dependent fashion by sodium orthovanadate and levamisole. And the IC_{50} was calculated to be 7.58 μM for sodium orthovanadate and 79.4 μM for levamisole, respectively, which were in good agreement with the values from previous reports.

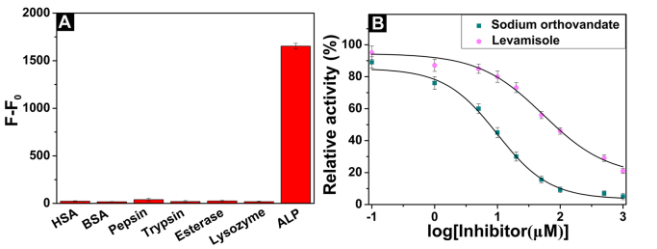


Fig. 4 (A) The fluorescence intensity changes of the probe **1** (10 μM) treated with different proteins (HSA, BSA, pepsin, trypsin, esterase, lysozyme and ALP) in 10 mM Tris-HCl buffer (pH 7.4); (B) relative activity of ALP (0.1 U/mL) as a function of different concentrations of ALP inhibitors (sodium orthovandate and levamisole) in 10 mM Tris-HCl buffer (pH 7.4).

After confirming the good stability and biocompatibility (Fig. S19-S21), and high specificity of probe **1** towards phosphatase, we then evaluated the potentials of probe **1** for real-time monitoring endogenous phosphatase activities in living cells. HeLa cells and HEK 293T cells was used as phosphatase positive control and phosphatase negative control, respectively, because of different expression level of ALP inside cells.[16] From the confocal fluorescence microscopy images displayed in Fig. 5, we found that HeLa cells treated with 10 μM of probe **1** clearly showed strong red fluorescence inside cells after incubation in 5 min. In contrast, no obvious fluorescence signals inside HEK 293T cells was observed at the same condition, indicative of the trace expression level of phosphatase inside the HEK 293T cell. In addition, when the HeLa cells were treated by both levamisole and probe **1**, no meaningful fluorescence signals inside the cells was observed from confocal microscopy images (Fig. S22), due to the proper inhibitory effect of levamisole on ALP inside cells. These results support that the enhanced fluorescent signals in HeLa cells were ascribed to the enzymatic activity of phosphatase.

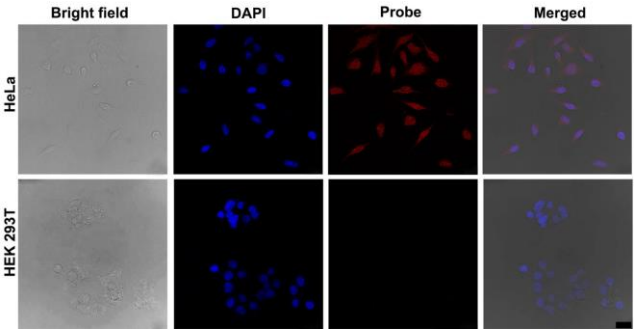


Fig. 5 Fluorescence microscopy images of HeLa cell and HEK 293 cells incubated with probe **1** (10 μM) for phosphatase imaging. Scale bar = 50 μm.

With the application of flow cytometry, we conducted quantitative analysis of phosphatase activity at single cell level. From the results shown in Fig. 6, we found that HeLa cells treated by probe **1** alone showed an obvious shift in their histogram compared to unstained HeLa cells and HeLa cells with inhibited ALP activities by levamisole, with different geometric mean fluorescence intensities at 222, 8.30 and 8.55, respectively. In addition, HEK 293T cells with low expression level of ALP also showed a distinct histogram when treated with probe **1** (Fig. 6), resulting in ca. more than 3-fold higher mean fluorescence intensity (i.e., 13.8) than that of unstained HEK 293T cells (i.e., 3.8) and 16-fold lower mean fluorescence intensity than that of stained HeLa cells (e.g., 222). These results above clearly demonstrated the high sensitivity and specificity of probe **1** towards phosphatase inside living cells, implying its potential for rapid and quantitative analysis of phosphatase activity at single cell level.

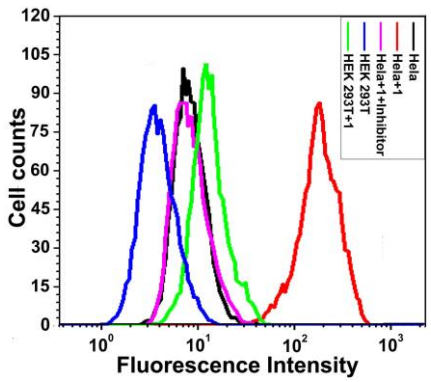


Fig. 6 Flow cytometry analysis of endogenous ALP activity in HeLa cells and HEK 293T cells with probe **1** at single cell level (E_x: 488 nm, E_m: 550–600 nm).

In summary, we have developed a novel probe for ALP detection from simple conjugation of a phosphate moiety to resorufin via a reactive *p*-hydroxybenzyl group. Because of its high enzymatic efficiency, good biocompatibility and rapid cell internalization, this probe exhibited great potential for ALP inhibitor screenings and real-time monitoring of intracellular phosphatase activities. We expect that our study can stimulate the design and development of novel probes with fluorescence turn-on property for monitoring the biological activities of phosphatase in living systems.

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Notes and references

[a] College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou, China 215123.
E-mail: xinmingli@suda.edu.cn.
[b] Department of Biochemistry and Molecular Biology, Medical College of Soochow University, Suzhou, China 215123.
Email: linguo@suda.edu.cn

Electronic Supplementary Information (ESI) available: [Detailed procedures for the synthesis of compound **1**, **2**, **3**, **4** and **5** and their corresponding NMR and MS spectra]. See DOI: 10.1039/c000000x/

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