# Analytical Methods

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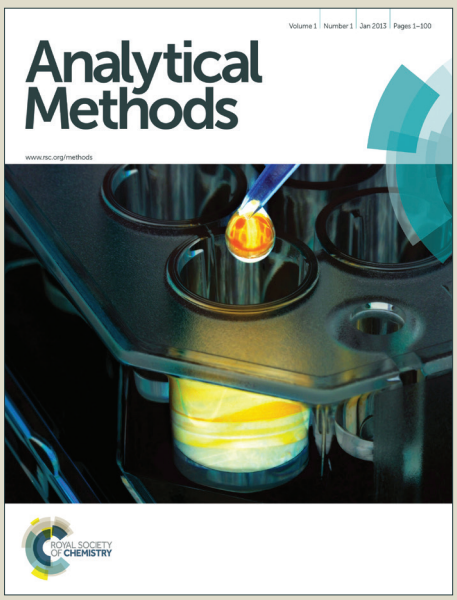





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ARTICLE TYPE

# A facile method for alkaline phosphatase activity detection based on the turn-on fluorescence of resorufin

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In this work, a facile fluorescence turn-on approach for the detection of alkaline phosphatase (ALP) activity has been developed. L-ascorbic acid-2-phosphate (AAP), a substrate of ALP, could be hydrolyzed by ALP to give L-ascorbic acid. L-ascorbic acid reduced resazurin to resorufin, which resulted in a turn on fluorescence signal. The fluorescence intensity increase could be directly related to the amount of ALP added to the assay solution. The assay is very sensitive, 0.12 mU/mL ALP could be clearly detected. Sodium orthovanadate ($Na_3VO_4$), a well known ALP inhibitor, was tested, and clear inhibition effect was observed. The results suggest that our method could be used for ALP activity sensing related various biochemical applications, and for the screening of ALP inhibitors.

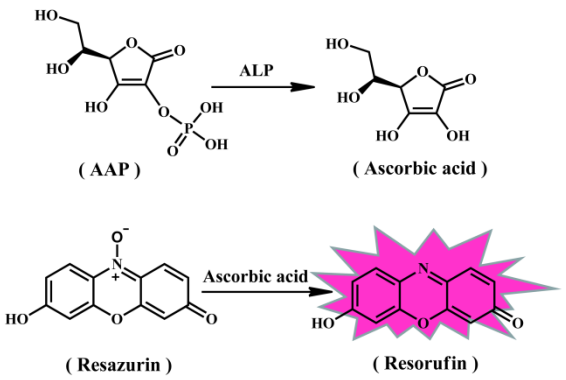
## Introduction

Alkaline phosphatase (ALP) is a common hydrolase in human tissues. It can catalyze the hydrolysis and transphosphorylation of a wide variety of phosphoric acid monoesters, including biological macromolecules and small organic molecules.[1] ALP plays a vital role in a variety of biological functions, such as the phosphorylation/dephosphorylation related cellular regulation and signaling processes. The abnormal level of ALP in human body is a signal for a variety of disease states involving particularly the liver and the bone.[2] Therefore, there is an increasing interest to explore new ALP detection methods with good sensitivity and selectivity.

Many techniques have been developed for the detection of ALP, such as the colorimetric,[3] electrochemical,[4] chromatographic,[5] chemiluminescent,[6] surface-enhanced Raman scattering,[7] and fluorometric methods.[8] Among these assays, fluorometric methods have attracted considerable interest for their rapid response, easy operation and adequate sensitivity. There are a variety of fluorescent probes, such as organic dyes,[9] conjugated polyelectrolytes,[8d,10] and quantum dots.[8a-b,11] And many novel fluorescence turn on sensing strategies have been developed in recent years.[12]

L-Ascorbic acid 2-phosphate (AAP) is a phosphatase substrate.[13] The non-reducing AAP is hydrolyzed in the presence of phosphatase, and the reductive ascorbic acid is generated in situ. Resazurin is a blue dye and has been used as a redox indicator since the 1950s to monitor the viability of microbial and mammalian cells, assess semen quality and cytotoxicity of anticancer drugs, etc.[14] Resazurin is weakly fluorescent, however, in the presence of a proper reducing reagent, it could be reduced to the pink color and highly fluorescent resorufin.[15]

Herein, a new fluorescence turn-on ALP detection approach is reported. AAP could be hydrolyzed by ALP and give ascorbic acid, resazurin could be reduced by ascorbic acid, the fluorescent resorufin is generated in situ, and the emission intensity increase of resorufin could be directed related to the amount of ALP added in the assay solution (Scheme 1). Our method is simple, sensitive and selective and could be used for the ALP sensing related various biochemical applications.




**Scheme 1.** Schematic illustration of the ALP assay principle.

## Experiment Section

### Reagents and Chemicals

Resazurin (sodium salt, HPLC purified for cell culture) was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). L-Ascorbic acid 2-phosphate and trypsin were obtained from Sigma-Aldrich Trading Co., Ltd. (St. Louis, MO, USA). Alkaline phosphatase (from calf intestinal) was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Bovine serum albumin (BSA) and lysozyme were purchased from Bio Basic Inc. (BBI, Markham, Canada). Sodium orthovanadate ($Na_3VO_4$) was

purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals were of analytical reagent grade. Water was purified with a Milli-Q system (Millipore, MA, USA).

### Instrumentation

UV-vis absorption spectra were obtained with a Cary 50 Bio spectrophotometer (Varian Inc., CA, USA). Fluorescence measurements were conducted using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc., USA). Sample solutions were excited at 530 nm and the fluorescence emission spectra were recorded with slits for excitation and emission both of 2 nm. A PB-10 pH meter (Sartorius Scientific Instrument Co., Ltd. Beijing) was used to adjust the pH of all buffer solutions. KQ-100DE model ultrasonic cleaning instrument (Kunshan Ultrasonic Instrument Co., Ltd.) was used to deaerate the water.

### Assay procedures

6.25 mM resazurin and 12.5 mM AAP aqueous solutions were stored at 4 ºC. Prior to the fluorescence measurements, the corresponding amount of resazurin solution, AAP solution, water and ALP were mixed with 2 × 20 mM Tris-HCl buffer (pH 7.5). The final assay solution contained 20 μM resazurin, 500 μM AAP, and different concentrations of ALP in 20 mM Tris-HCl buffer (pH 7.5). The assay solution was then incubated at 37 ºC for an appropriate period of time, and the emission intensity changes were monitored.

The inhibition efficiency (IE) was defined by the following equation:

$$IE = [IF_{(\text{no inhibitor})} - IF_{(\text{inhibitor})}]/[IF_{(\text{no inhibitor})} - IF_0]$$

in which $IF_{(\text{inhibitor})}$ and $IF_{(\text{no inhibitor})}$ refer to the emission intensity of resorufin in the presence or absence of inhibitors, and $IF_0$ refers to background emission of resorufin.

ALP detection in serum samples: the assay solutions contained 20 μM resazurin, 500 μM AAP, and different concentrations of ALP in 20 mM Tris-HCl buffer (pH 7.5) containing 0.2% fetal bovine serum. Samples were incubated at 37 ºC for 60 min and the emission spectra were taken.

## Results and discussion

The reduction reaction of resazurin by ascorbic acid was examined (Figure 1). 2.5 mM ascorbic acid was added to 20 μM resazurin, the intensity of the UV-vis absorption bands of resazurin at 380 nm and 600 nm decreased gradually, and the absorption band of resorufin at 571 nm appeared and the intensity became stronger. The color of the assay solution also changed from blue to pink. In addition, a fluorescence emission band with peak maximum at 583 nm appeared. The significant changes in the UV-vis and emission spectra clearly suggest the conversion of resazurin to resorufin. The changes in assay solution color and the appearance of strong emission of resorufin are also in consistent with the conclusions.[16]

The ALP catalyzed AAP hydrolysis was examined (Figure 2).[4a-c,17] 20 μM resazurin was mixed with 250 μM AAP and 120 mU/mL ALP, and the emission intensity changes at 583 nm were monitored. The emission intensity increased gradually with the prolonged enzymatic reaction time, and reached a plateau after about 145 min of reaction. The emission intensity increase was quite significant, about a total of seven fold increase was observed. On the contrary, the emission spectra of the assay solutions containing resazurin only, or resazurin and AAP showed no obvious changes. The results clearly suggest that AAP was hydrolyzed by ALP and increasing concentrations of resorufin were produced with the increase of the enzymatic reaction time.

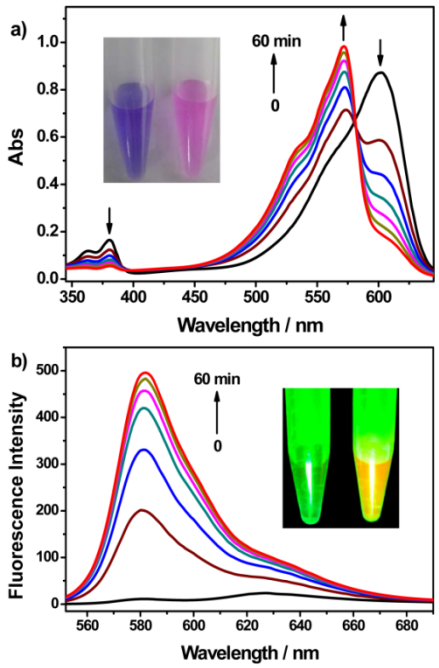



**Fig. 1** UV-vis (left) and emission (right) spectral changes. 2.5 mM ascorbic acid was mixed with 20 μM resazurin in 20 mM Tris-HCl buffer (pH 7.5), and the spectra were taken every 10 min. Insets: photographs of the corresponding reaction mixtures after 60 min reaction, left: under normal visible light, right: under 530 nm excitation.

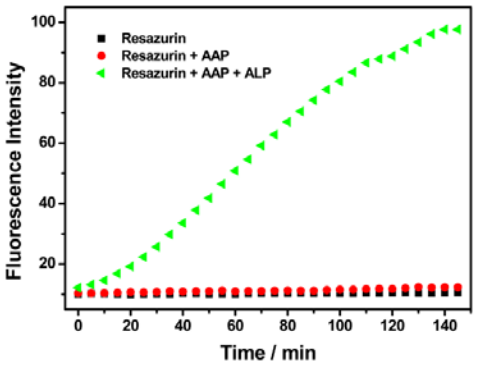



**Fig. 2** Changes in emission intensity with reaction time, and with or without the addition of ALP. The concentrations of resazurin, AAP, and ALP were 20 μM, 250 μM, and 120 mU/mL, respectively. Buffer: 20 mM Tris-HCl (pH 7.5). The solutions were excited at 543 nm and the emission intensities at 583 nm were recorded at 37 ºC.

The ALP assay was studied in detail. The real time emission intensity changes of the sample solutions at 583 nm and at different ALP concentrations (0, 0.6, 1.2, 2.4, 3.6, 6.0, 12, 24, and

120 mU/mL) were investigated. Fig. 3a shows that the emission intensity increased gradually with increasing ALP enzymatic reaction time. Larger degree of emission increase was observed with higher concentrations of ALP added. The emission intensity at 583 nm after 60 min reaction was plotted against the ALP concentration, and a linear relationship was obtained (Fig. 3b). The linear calibration curve is: IF = 6.45 + 1.837$C_{ALP}$ (IF refers to the emission intensity of resorufin, $C_{ALP}$ refers to the concentration of ALP added in the assay solution in mU/mL). The assay is quite sensitive, 0.6 mU/mL ALP gave a clear signal which could be easily distinguished from the background.

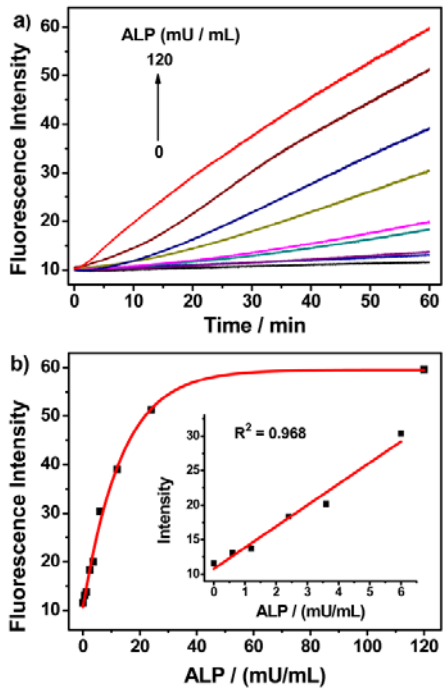



**Fig. 3** (a) Real-time emission intensity changes at 583 nm and at different ALP concentrations (0, 0.6, 1.2, 2.4, 3.6, 6.0, 12, 24, and 120 mU/mL). (b) Changes in emission intensity at 583 nm with ALP concentration. Reaction time: 60 min. Inset: linear relationship between the emission intensity at 583 nm and ALP concentration. Assay solutions contained 20 μM resazurin, 500 μM AAP, and different concentrations of ALP in 20 mM Tris-HCl (pH 7.5).

When the reaction time was extended further, better detection sensitivity was obtained. Fig. 4 shows the ALP concentration plotted against the emission intensity, a linear relationship was again obtained. The linear calibration curve is: IF = 11.06 + 16.96$C_{ALP}$, a lower detectable concentration of 0.12 mU/mL ALP was obtained. In addition, the enzymatic reaction could also be clearly monitored by the changes in assay solution color with the naked eyes (Inset b of Fig. 4). Our method is more sensitive than most of the recently reported methods for detecting ALP, including colorimetric methods,[3] fluorimetric methods[8,9a] as well as electrochemical methods.[4c-e]

The selectivity of the current assay was studied. A number of the commonly used proteins (trypsin, BSA, and lysozyme) were tested (Fig. 5). The results show that none of these proteins gave significant fluorescence increase, which clearly indicate that these proteins did not interfere with our assay. The influence of metal ions and anions were also tested, the results show that they do not show noticeable interference on the ALP assay (Fig. S1, Supporting Information).

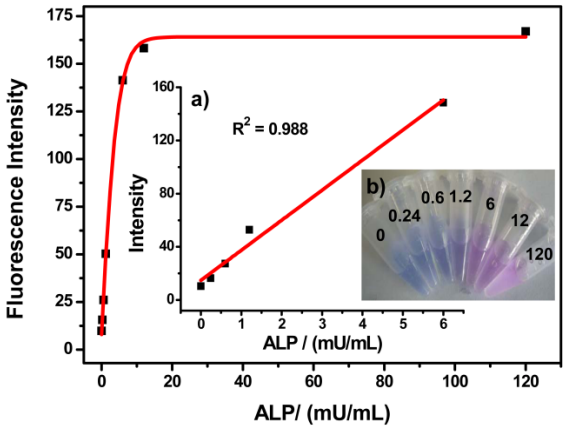



**Fig. 4** Emission intensity changes at 583 nm with ALP concentration. Inset: (a) linear relationship between the emission intensity and the ALP concentration, (b) photograph of the corresponding samples. All samples contained 20 μM resazurin, 500 μM AAP, and different concentrations of ALP. Incubation time: 5 hours at 37 ºC in 20 mM Tris-HCl (pH 7.5).

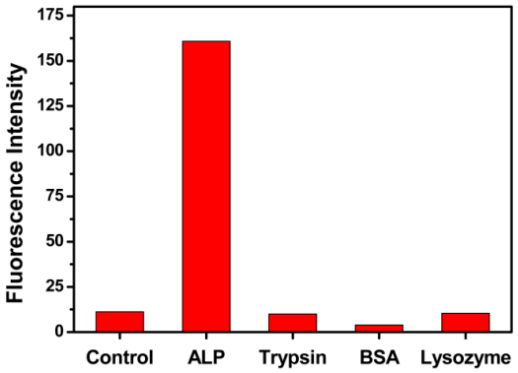



**Fig. 5** Selectivity study. All samples contained 20 μM resazurin, 500 μM AAP, and incubated at 37 ºC for 5 hours. Concentrations of the proteins: 120 mU/ml ALP; 37 μM BSA; 660 U/ml (17 μM) trypsin; and 8 kU/ml (29 μM) lysozyme. Control: blank sample with no ALP or the other proteins added.

The degree of AAP hydrolysis by ALP could be reduced when an inhibitor of ALP is introduced. And reduced fluorescence intensity increase would be expected. A common ALP inhibitor, namely sodium orthovanadate ($Na_3VO_4$), was selected and tested. The real-time emission intensity changes at 583 nm were monitored in the presence of different concentrations of $Na_3VO_4$. And the inhibition efficiency was calculated (Fig. 6).[9a] The degree of fluorescence increase reduced significantly with the increase of $Na_3VO_4$ concentration. The results suggest that the rate of ALP hydrolysis decreased gradually at increasing $Na_3VO_4$ concentrations. The $IC_{50}$ value (the concentration of an inhibitor required to achieve 50% of inhibition efficiency) of $Na_3VO_4$ was estimated to be 148.6 μM. The results indicate that our method could be utilized for the screening of ALP inhibitors.

The assay was also tested in diluted serum samples (0.2% fetal

bovine serum). Fig. S2 (Supporting Information) shows that with the addition of increasing ALP concentrations, the emission intensity of resorufin at 583 nm gradually increased. Standard recovery assay was also performed, and satisfactory recovery values were obtained (Table S1, Supporting Information).

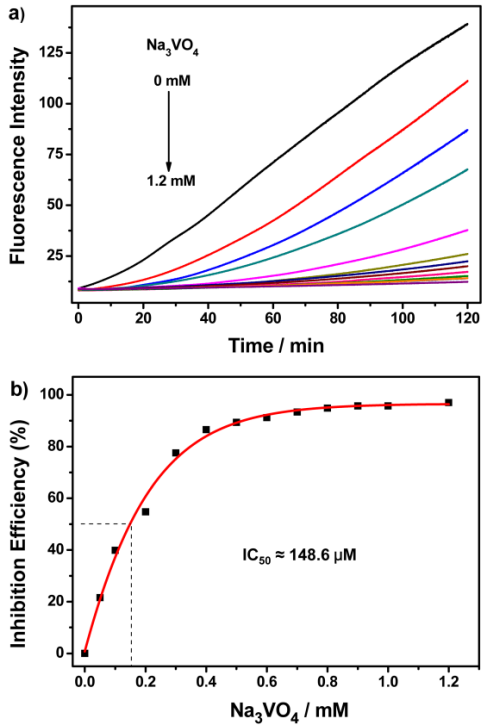



**Fig. 6** (a) Real-time emission intensity changes at different $Na_3VO_4$ concentrations. (b) Plot of the inhibition efficiency versus $Na_3VO_4$ concentration. Samples contained 20 μM resazurin, 120 mU/ml ALP, and 500 μM AAP in 20 mM Tris-HCl (pH 7.5). Emission intensity changes were monitored at 583 nm.

## Conclusions

In summary, a facile real-time ALP activity assay method has been developed. ALP catalyzed the hydrolysis of a substrate (AAP) and ascorbic acid was generated in situ. Ascorbic acid could effectively reduce the very weakly fluorescent resazurin to the highly fluorescent resorufin, and a turn on fluorescence signal was detected. The emission intensity increase could be directly related to the amount of ALP added to the assay solution. Our assay is highly sensitive, 0.12 mU/mL ALP could be clearly detected. The assay is also quite selective, a number of proteins were tested, and none gave noticeable interference. A well known ALP inhibitor ($Na_3VO_4$), was tested, and clear inhibition effect was observed. The results suggest that our method could be used for the sensing ALP activity and for the screening of ALP inhibitors in related biological and biochemical applications.

## Acknowledge

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## Notes and references

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