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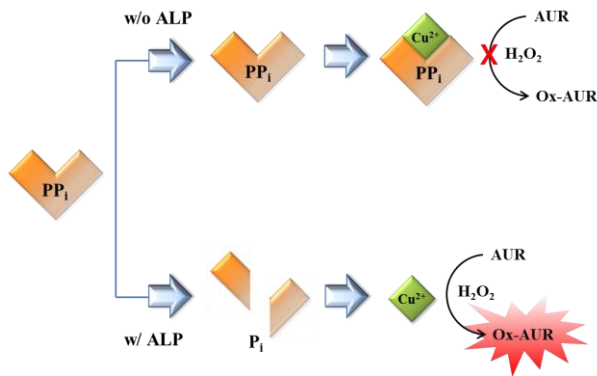
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A sensitive dual colorimetric and fluorescence system for assaying the activity of alkaline phosphatase that relies on pyrophosphate inhibition of the peroxidase activity of copper ions

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A novel and highly sensitive optical assay for the accurate determination of alkaline phosphatase activity is developed by utilizing the peroxidase activity of Cu^{2+} ions.



ARTICLE

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Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

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A novel and highly sensitive colorimetric and fluorescence assay for the accurate determination of alkaline phosphatase (ALP) activity has been developed. The assay takes advantage of the inhibition of the peroxidase activity of Cu^{2+} ions caused by complexation with pyrophosphate (PPi), a natural substrate for ALP. This inhibition disappears when PPi undergoes ALP catalyzed hydrolysis to generate phosphate, which does not bind to Cu^{2+} ions. Thus, ALP causes generation of uncomplexed Cu^{2+} ions, which promote multiple oxidations of Amplex UltraRed in the presence of hydrogen peroxide in conjunction with the production of intense fluorescence and colorimetric signals. By employing the fluorescence and colorimetric assay strategies, ALP can be detected at respective concentrations as low as 4.3 pM and 5.4 pM, detection limits that are much lower than those associated with previously described methods. The practical diagnostic capability of the assay system has been demonstrated by its use to detect ALP in human blood serum.

Introduction

Phosphatases are enzymes that promote hydrolytic removal of the phosphate group of phosphate monoesters to produce phosphate and products containing free hydroxyl groups. As a result, these enzymes play pivotal roles in signal transduction and regulation of intracellular processes involved in cell cycle, growth, apoptosis and signal transduction pathways.¹⁻⁴ The most common human alkaline phosphatase, ALP, is found in all tissues throughout the body but it is mainly located in the liver, bile duct, kidney, bone, and placenta.⁵ Owing to the fact that altered levels of ALP are closely linked to various diseases, such as breast and prostatic cancer, bone disease, diabetes, hepatitis, and liver dysfunction,⁵⁻⁷ determination of the activity of this enzyme is highly significant in clinical diagnoses of these diseases. In addition, as a consequence of its high turnover number and stability, low cost, and broad substrate specificity, ALP is one of the most widely used labeling enzymes in affinity-based biosensors.⁸⁻¹⁰

At present time, ALP activity is typically assayed by using several commercially available kits, which employ specially designed substrates such as *p*-nitrophenyl phosphate, 4-methylumbelliferyl phosphate and *p*-aminophenyl phosphate. These substrates are readily hydrolyzed by ALP to produce yellow colored (*p*-nitrophenol), highly fluorescent (4-methylumbelliferone) or electroactive (*p*-aminophenyl) products, whose quantitative detection employing spectroscopic or electrochemical methods is employed to determine ALP activity.¹¹⁻¹⁶ Other methods, devised to determine ALP activity, employ pyrophosphate (PPi), a natural substrate of this enzyme which is hydrolyzed in the human body to form inorganic phosphate as part of skeletal mineralization and vascular

calcification processes.¹⁷⁻¹⁹ Using these methods, the more direct information about the relationship between ALP and PPi could be obtained. More recently, Schanze et al. described a new, conjugated polyelectrolyte based fluorescence method for assaying ALP. The system takes advantage of reversible binding of Cu^{2+} to PPi and carboxylate groups in the conjugated polymer^{6,20} and the fact that ALP converts PPi to phosphate (Pi), a process that liberates uncomplexed Cu^{2+} , which then binds to and quenches the fluorescence of the conjugated polymer. Although the method developed by Schanze is both straightforward and rapid, it suffers from potential false positive signals as a consequence of its turn-off signaling nature.²¹⁻²³

Detection systems that display turn-on fluorescence responses in the presence of ALP have also been designed. One approach of this type is based on the formation of fluorescent copper nanoparticles employing double-stranded DNA as a template. In this system, ALP hydrolyzes PPi and releases free Cu^{2+} ions, which effectively form the DNA-templated fluorescent copper nanoparticles.¹⁷ Unfortunately, the detection sensitivity of this sensing system is not high enough to be used in a practical manner. Another strategy, which utilizes ALP to control PPi regulated Zn^{2+} dependent DNzyme activity, is highly sensitive but it requires potentially costly chemical modification of a DNA probe to introduce the fluorophore and quencher.²⁴ Furthermore, the use of a chimeric DNA (oligonucleotide containing at least one ribonucleotide base) in the sensing system could be detrimental owing to the high susceptibilities of RNA molecules to degradation.²⁵

As a result of the fact that systems developed thus far for the determination of ALP activity have less than ideal characteristics, we have conducted studies aimed at devising a

simple, cost effective and highly sensitive method for this purpose. This investigation led to a dual colorimetric and fluorescence based system for assessing ALP activity, which takes advantage of several key features. The first involves the known oxidation reaction of Amplex UltraRed (AUR) by free Cu^{2+} in the presence of hydrogen peroxide (H_2O_2) to produce a highly fluorescent and intensely colored product.²⁶ In addition, the technique relies on the finding that the ability of Cu^{2+} to promote multiple oxidations of AUR is significantly suppressed by chelation with PPI. The final conceptual component of the new assay system is that PPI inhibition of Cu^{2+} promoted oxidation of AUR is obliterated in the presence of ALP, which hydrolytically cleaves PPI to form Pi. The studies based on this design principle, described below, have led to the development of a new, simple and highly sensitive ALP assay procedure, which does not suffer from disadvantages associated with previously devised methods.

Experimental Section

Materials: Copper(II) nitrate, sodium pyrophosphate, sodium phosphate (monobasic & dibasic), hydrogen peroxide, tris(hydroxymethyl) aminomethane, acetic acid, alkaline phosphatase, lysozyme, avidin, glucose oxidase, thrombin, bovine serum albumin, and human serum were purchased from Sigma-Aldrich. AUR (Amplex® UltraRed) reagent with the molar absorptivity of $11,000 \pm 1,000 \text{ cm}^{-1}\text{M}^{-1}$ was purchased from Invitrogen (Catalog no. A36006, Eugene, OR, USA). All other chemicals were of analytical grade and used without purification.²⁷⁻²⁹ Aqueous solutions were prepared using ultrapure DNase/RNase-free distilled water purchased from Bioneer® (Daejeon, Korea). The Tris-acetate buffer (10 mM, pH 7) was used as the reaction buffer throughout this study. The pH of the reaction buffer was chosen to be 7 in the overall experiments because the peroxidase activity of Cu^{2+} ions was reported to be optimal at pH 7.²⁶ At lower pH values, AUR is difficult to be oxidized and at higher pH values, undesirable species such as $\text{Cu}(\text{OH})_2$ are formed, which may lead to the reduction of the peroxidase activity of Cu^{2+} ions.²⁶

Optimization of the peroxidase activity of Cu^{2+} ions: Solutions (5 μL) of varying concentrations of Cu^{2+} ions, 5 μL of AUR (500 μM) and 5 μL of H_2O_2 (10 mM) of a total volume of 50 μL in 10 mM Tris-acetate (pH 7) were incubated and the intensities of their fluorescence signals were recorded after standing for 20 min at room temperature.

Determination of the effects of Pi and PPI on the peroxidase activity of Cu^{2+} ions: Solutions (5 μL) of varying concentrations of PPI or Pi (H_2PO_4^- and HPO_4^{2-}) (2 mM) and 5 μL of Cu^{2+} ions (1 mM) were mixed making a total volume of 40 μL in 12.5 mM Tris-acetate (pH 7) and then incubated at room temperature for 20 min. A solution (5 μL) of AUR (500 μM) and 5 μL of H_2O_2 (10 mM) were added to the above solutions (total volume of 50 μL) in 10 mM Tris-acetate (pH 7). The intensities of the fluorescence and absorption signals of the solutions were determined 20 min after addition of AUR and H_2O_2 .

ALP detection procedure: Solutions (5 μL) of varying concentrations of ALP or other proteins, such as lysozyme, avidin, glucose oxidase, thrombin and bovine serum albumin (100 nM) and 5 μL of PPI (1 mM) were mixed making a total volume of 35 μL in 14.3 mM Tris-acetate (pH 7) and then incubated at 37 °C for 60 min. A solution (5 μL) of Cu^{2+} ions (1 mM) was then added and the resulting solution was incubated at room temperature for 20 min. A solution (5 μL) of AUR (500 μM) and 5 μL of H_2O_2 (10 mM) were then added to yield a total volume of 50 μL in 10 mM Tris-acetate (pH 7). The intensities of the fluorescence and absorption signals were recorded 20 min after the addition of AUR and H_2O_2 .

ALP detection procedure in human serum: Human serum (10 μL) was diluted with 80 μL of 10 mM Tris-acetate (pH 7) and then spiked with 10 μL of solutions containing varying concentrations of ALP. Aliquots (5 μL) of the diluted human serum solutions (10 %) containing ALP were mixed with 5 μL of PPI (1 mM) to give a total volume of 35 μL in 14.3 mM Tris-acetate (pH 7), and incubated at 37 °C for 60 min. The samples were submitted to the same fluorescence and colorimetric analysis methods described above.

Instrumentation: Fluorescence intensities ($\lambda_{\text{ex}} = 540 \text{ nm}$) and UV-visible absorption intensities were determined using a Tecan Infinite M200 pro microplate reader (Mnndorf, Switzerland).³⁰ Fluorescence measurements were conducted with undiluted samples and the absorption intensities were determined using solutions that were diluted with 45 μL of the assay solution and 55 μL of water.

Results

The conceptual design of the new assay system to assess ALP activity is illustrated in Fig. 1. The procedure utilized in this approach involves initial incubation of PPI with the sample being analyzed followed by the sequential addition of Cu^{2+} and the signal generation reagents comprised of AUR and H_2O_2 . When ALP is present in the test sample, hydrolysis of PPI occurs to generate Pi, making it unavailable to complex with Cu^{2+} . As a result, the peroxidase activity of free Cu^{2+} causes multiple oxidation reactions of AUR in the presence of H_2O_2 . Thus, the presence of ALP in the test sample is signaled by the production of an intense naked eye detectable color and strong fluorescence of the product formed by AUR oxidation.

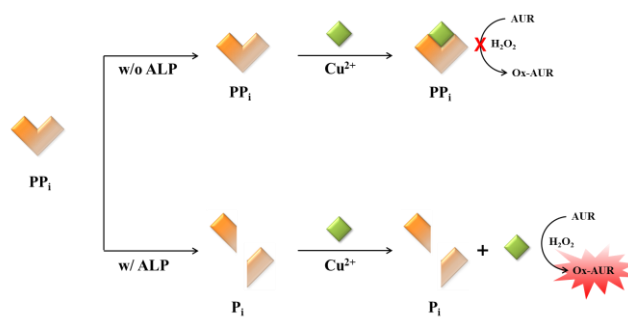


Fig. 1 Schematic illustration of fluorescence and colorimetric ALP assay based on PPI inhibition of the peroxidase activity of copper ions.

In the initial phase of the study probing the feasibility of the new ALP assay, optimal conditions for the peroxidase activity of Cu^{2+} were explored by examining the intensities of the fluorescence signals arising from AUR oxidation. The results of experiments, in which Cu^{2+} concentrations and incubation times were varied, demonstrate that 100 μM Cu^{2+} and a 20 min incubation time are ideal to achieve optimal emission signals (Fig. S1 and S2, ESI†).

The effects of Pi and PPI on the peroxidase activity of Cu^{2+} were examined next. As shown in Fig. 2, free Cu^{2+} promotes multiple oxidations of AUR in association with the production of an intense fluorescence signal at 590 nm and an intense UV-visible absorption band at 570 nm (1, Fig. 2). The presence of Pi (H_2PO_4^- and HPO_4^{2-}) does not lead to inhibition of the reaction between Cu^{2+} and AUR and, consequently, it has no effect on the generation of fluorescence and color (2, 3 and 4, Fig. 2). However, PPI, which tightly binds Cu^{2+} , completely suppresses the peroxidase activity of Cu^{2+} and, as a result, it causes a large decrease in AUR oxidation and the formation of fluorescence and absorption bands (5, Fig. 2). Most importantly, the intensities of the fluorescence and colorimetric signals produced

by Cu^{2+} promoted oxidation are nearly totally regenerated when ALP is first added to a test solution containing PPI (6, Fig. 2). These observations clearly demonstrate that free Cu^{2+} displays peroxidase activity towards AUR and this activity is suppressed by complexation of Cu^{2+} with PPI.

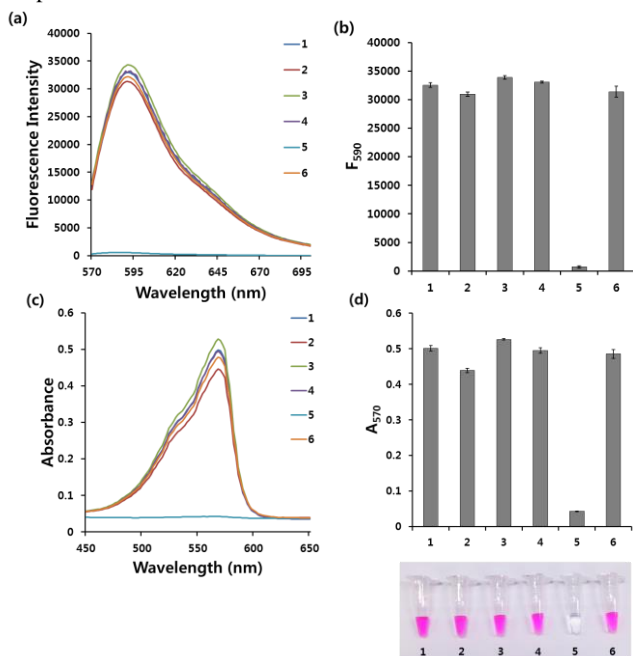


Fig. 2 (a) Fluorescence and (c) UV-vis absorption spectra of solutions containing free Cu^{2+} (1), Cu^{2+} together with 200 μM H_2PO_4^- (2), 200 μM HPO_4^{2-} (3), a mixture of 100 μM H_2PO_4^- and 100 μM HPO_4^{2-} (4), and 100 μM PPI (5) or 100 μM PPI treated with 1 nM ALP (6). (b) Corresponding fluorescence intensities at 590 nm and (d) absorption intensities at 570 nm. Inset: Photographic images of the solutions. The final concentrations of Cu^{2+} , AUR and H_2O_2 are 100 μM , 50 μM and 1 mM, respectively.

The optimal concentration of PPI required to block Cu^{2+} promoted generation of fluorescence was determined next. For this purpose, fluorescence intensities at 590 nm, arising from the product formed by Cu^{2+} catalyzed oxidation of AUR, in the presence of varying concentrations of PPI were determined. Analysis of the spectra and plots displayed in Fig. 3 shows that fluorescence intensities decrease linearly with increasing concentrations of PPI in the range of 0–100 μM . However, the extent of the reduction reaches a minimum value at PPI concentrations >100 μM . Because of this saturation phenomenon and the possibility that excessively high concentrations of PPI might cause non-productive hydrolysis by ALP, which would have a negative impact on detection sensitivity, 100 μM PPI was used in the assay system.

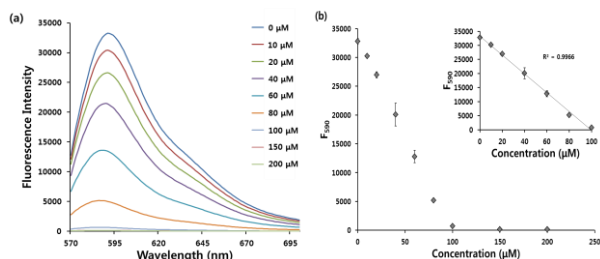


Fig. 3 (a) Fluorescence spectra of oxidation products formed by treatment of AUR with Cu^{2+} in the presence of varying concentrations of PPI. (b) PPI concentration dependent reduction of fluorescence intensity at 590 nm. Inset: The linear range of the plot between F_{590} and the PPI concentration (0–100 μM). The final concentrations of Cu^{2+} , AUR and H_2O_2 are 100 μM , 50 μM and 1 mM, respectively. See the enlarged version in Fig. S3, ESI†.

In further studies aimed at defining ideal assay conditions, we observed that ALP treatment of a premixed mixture of PPI and Cu^{2+} leads to only 40% recovery (3, Fig. S4, ESI†) of the fluorescence signal that is displayed when PPI is absent (1, Fig. S4, ESI†). This finding indicates that PPI bound to Cu^{2+} is not as efficiently hydrolyzed by ALP as is free PPI, presumably because of the hydrolytic inhibitory effect of Cu^{2+} .³¹ In contrast, near complete recovery of the peroxidase activity of Cu^{2+} takes place when it is added to a mixture of PPI and ALP (4, Fig. S4, ESI†). Therefore, the ideal detection assay procedure involves addition of PPI to the test sample containing ALP followed by incubation and addition of Cu^{2+} .

In order to show that ALP catalyzed hydrolysis of PPI is the process responsible for recovery of fluorescence and colorimetric signals, the effects of other enzymes and proteins were determined and compared with that of ALP. The data in Fig. 4 demonstrate that PPI inhibition of the peroxidase activity of Cu^{2+} is effectively suppressed when 1 nM ALP is present and not when higher concentrations (10 nM) of other enzymes and proteins are used.

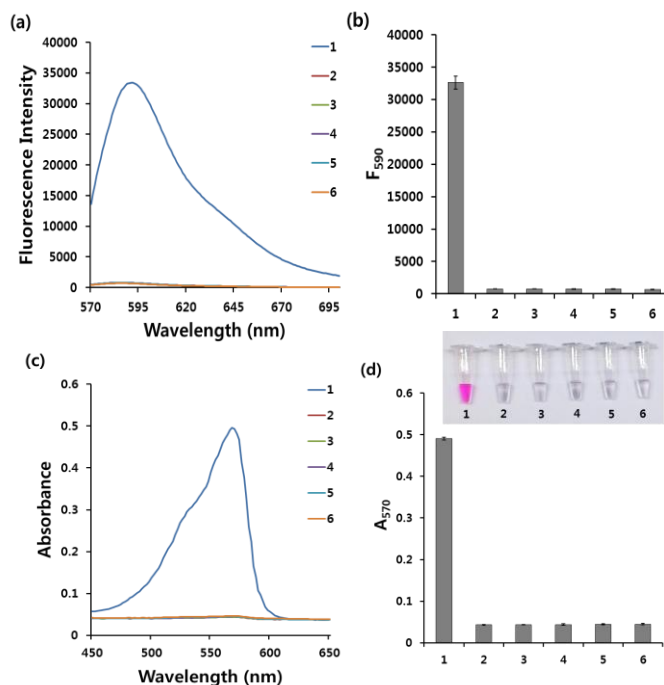


Fig. 4 (a) Fluorescence and (c) UV-vis absorption spectra of the oxidation products of the reaction between AUR and Cu^{2+} in the presence of 1 nM ALP (1) and 10 nM of other proteins such as lysozyme (2), avidin (3), glucose oxidase (4), thrombin (5), and bovine serum albumin (6). (b) Corresponding fluorescence intensities at 590 nm and (d) absorption intensities at 570 nm. Inset: Photographic images of the solutions. The final concentrations of Cu^{2+} , PPI, AUR substrate and H_2O_2 are 100 μM , 100 μM , 50 μM and 1 mM, respectively.

The detection sensitivity of the new ALP assay was determined by measuring fluorescence intensities at 590 nm and absorption intensities at 570 nm as a function of ALP concentrations. The results show that fluorescence intensities increase with increasing concentrations of ALP up to 500 pM, and then plateau at concentrations >500 pM (Fig. 5). An excellent linear relationship ($R^2 = 0.9931$) exists in this plot in the range of 0–500 pM and the limit of detection (LOD) ($3\sigma/\text{slope}$) is ca. 4.3 pM. Importantly this LOD value is much lower than those reported for other fluorescence based methods for ALP detection.^{6,7,24,32–35} The absorption intensities display a similar concentration dependence with the same linear range of 0–500 pM and an LOD of ca. 5.4 pM, a value that is also

much lower than those reported for other colorimetric ALP detection methods (Fig. 5).³⁶⁻³⁸

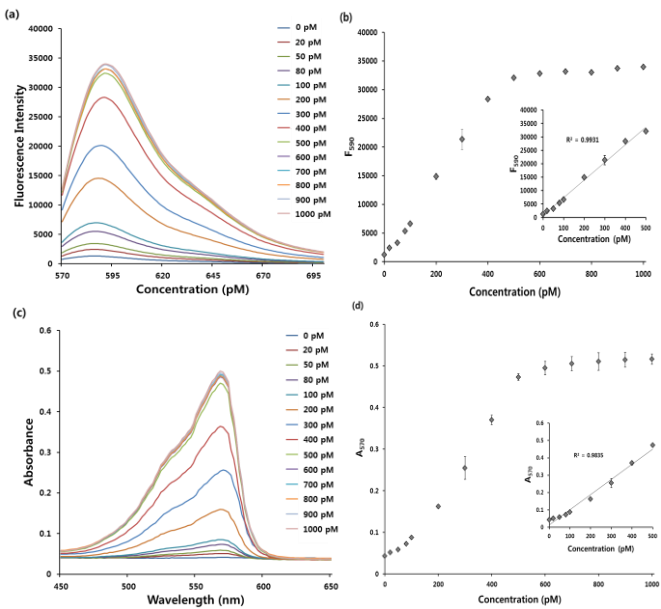


Fig. 5 (a) Fluorescence and (c) UV-vis absorption spectra of oxidation products of the reaction between AUR and Cu²⁺ in the presence of varying concentrations of ALP. (b) ALP concentration dependent change of fluorescence intensities at 590 nm. Inset: The linear range of the plot between F₅₉₀ and ALP concentration (0–500 pM). (d) ALP concentration dependent change of absorption intensity at 570 nm. Inset: The linear range of the plot between A₅₇₀ and ALP concentration (0–500 pM). The final concentrations of Cu²⁺, PPi, AUR substrate and H₂O₂ are 100 μM, 100 μM, 50 μM and 1 mM, respectively. See the enlarged version in Fig. S5, ESI†.

A practical application of the new assay system was demonstrated by its utility in detecting ALP in human serum. As shown by inspection of Fig. S6, the pattern of the fluorescence response to ALP added to diluted human serum (1 %) is nearly the same as that from an artificially constructed solution containing only ALP (Fig. 5 (a) and (b)). Specifically, the fluorescence intensities at 590 nm increase with increasing concentrations of ALP, spiked into diluted human serum, and a good linear correlation exists in the range of 0–500 pM (Fig. S6, ESI†). The excellent reproducibility and precision of the assay system is evidenced by a coefficient of variation (CV) that is less than 8% and recovery ratio between 97 and 105%. These observations show that the ALP assay procedure has the potential of being used reliably to determine the amounts of ALP present in human serum samples (Table 1).

Table 1. Determination of ALP in spiked human serum.

Added ALP (pM)	Measured ALP (pM) ^{a)}	SD ^{b)}	CV ^{c)} (%)	Recovery ^{d)} (%)
150	157.4	12.10	7.69	104.9
250	246.1	9.09	3.69	98.4
350	363.9	6.38	1.75	104.0
450	438.5	23.09	5.27	97.4

^{a)} To measure the amount of ALP, a calibration curve was first created by using standards containing a known amount of ALP in human serum (Fig. S6, ESI†). Using this calibration curve, the fluorescence intensities from the unknown samples were used to determine the concentration of ALP present in human serum.^{17,21,39}

^{b)} Mean of three measurements.

^{c)} Standard deviation of three measurements.

^{d)} Coefficient of variation = SD/mean × 100.

^{e)} Measured value/added value × 100.

Conclusions

In the investigation described above, we developed a novel, dual colorimetric and fluorescence turn-on assay system for highly sensitive detection of ALP. The method employs PPi, a natural ALP substrate, and takes advantage of PPi induced suppression of the fluorescence and color forming reaction of Cu²⁺ with AUR. Importantly, the peroxidase activity suppression is obliterated by ALP because it catalyzes the hydrolysis of PPi to form Pi. This method can be conveniently applied to ALP analysis because it does not require complicated chemical modifications of assay components. In addition, the method gives rise to colorimetric and fluorescence signals that linearly correlate with ALP concentrations. Finally, the technique has ALP detection limits of 4.3 and 5.4 pM in a turn-on fluorescence and colorimetric manner, respectively, which are significantly lower than those previously reported. The assay system developed in this effort contains the first successful application of the peroxidase activity of Cu²⁺ to the determination of enzyme activity. As such, the approach should pave the way for a more broad utilization of the catalytic activity of metal ions.

Acknowledgements

This study was supported by the grant from Public Welfare & Safety Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (MSIP) (No. 2012M3A2A1051683). Financial support was also provided by Center for BioNano Health-Guard funded by MSIP of Korea as Global Frontier Project (Grant H-GUARD_2013M3A6B2078964) and the Industrial Source Technology Development Program of the Ministry of Knowledge Economy (MKE) (No. 2010-10038662).

Notes and references

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†Electronic Supplementary Information (ESI) available. See DOI: 10.1039/c000000x/

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