Metal-to-Ligand Charge-Transfer-based Visual Detection of Alkaline Phosphatase Activity

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The ability to directly detect alkaline phosphatase (ALP) activity in undiluted serum samples is of great importance for clinical diagnosis. In this work, we report the use of the distinctive metal-to-ligand charge-transfer (MLCT) absorption properties of the $Cu(BCA)_2^+$ (BCA = bicinchoninic acid) reporter for the visual detection of ALP activity. In the presence of ALP, the substrate ascorbic acid 2-phosphate (AAP) can be enzymatically hydrolyzed to release ascorbic acid (AA), which in turn reduces Cu^{2+} to Cu^+. Subsequently, the complexation of Cu^+ with the BCA ligand generates the chromogenic $Cu(BCA)_2^+$ reporter, accompanied by a color change of colorless-to-purple of the solution with a sharp absorption band at 562 nm. The underlying MLCT-based mechanism has been demonstrated on the basis of density functional theory (DFT) calculations. Needless of any sequential multistep operations and elaborately designed colorimetric probe, the proposed MLCT-based method allows for a fast and sensitive visual detection of ALP activity within a broad linear range of 20 – 200 mU mL^{-1} (R^2 = 0.999), with a detection limit of 1.25 mU mL^{-1}. The results also indicate that it is highly selective and has great potential for the screening of ALP inhibitors in drug discovery. More importantly, it shows a good analytical performance for the direct detection of the endogenous ALP levels of undiluted human serum samples. Owing to the prominent simplicity and practicability, it is reasonable to conclude that the proposed MLCT-based method has a high application prospect in clinical diagnosis.

Keywords Alkaline phosphatase, bicinchoninic acid, colorimetric, metal-to-ligand charge transfer, serum

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Introduction

Alkaline phosphatase (ALP), a nonspecific phosphomonoesterase, is essential for the dephosphorylation- and phosphorylation-related metabolic processes of living organisms.[1,2] Since the abnormal levels of serum ALP have significant implications in hepatobiliary and bone diseases,[3–5] the ability to directly detect ALP activity in undiluted serum samples is of great importance for clinical diagnosis.

Up to now, a number of techniques have been developed to quantify ALP activity based on the catalytic property of dephosphorylation. Many methods have been proposed with variety techniques; for instance, chemiluminescence,[6] fluorescence,[7] surface-enhanced Raman spectroscopy,[8] and electrochemical technology.[9,10] Nevertheless, most of these methods requires complicated operation and expensive instrumentation. Besides, because the signal of some techniques is relatively weak, these methods may not meet the requirements of sensitivity to accurately determine the ALP level in human blood (40 – 190 U L^{-1} for adults), which limits the practical application in medical diagnosis. In comparison, by means of the colorimetric method, the ALP level can be directly observed with naked-eyes without any requirement of complicated and expensive instruments.[12] At present, phenyl phosphate and *p*-nitrophenyl phosphate (*p*NPP) have been widely used as substrates for detecting ALP activity in colorimetric methods. Estimating the serum ALP activity with phenyl phosphate was based on a method of King and Armstrong.[13] There are different ways to detect ALP activity by monitoring the concentration of phenol formed when ALP reacts with phenyl phosphate. For instance, phenol reacts with 4-aminoantipyrine in the presence of ferriccyanide to give a purple color with a short test time because of the high hydrolysis rate of phenyl phosphate.[14] However, the sensitivity and accuracy of this method cannot meet the requirements of modern medical treatment, and interfered by bilirubin and hemolysis. As for *p*NPP, it hydrolysis in the presence of ALP to proceed a yellow water-soluble reaction product, paranitrophenol (PNP), which absorbs strongly at 410 nm.[15,16] Owing to the convenience and efficiency of the *p*NPP-based method, it has been accepted as the standard for detecting ALP activity. However, the shortcoming of the *p*NPP-based method is obvious since its substrate is liable to hydrolysis under light, inducing a false positive result of the ALP level. For this reason, it is necessary to avoid light for *p*NPP in the process of preservation and detection.[17] Gao *et al.* designed a

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high-resolution colorimetric protocol based on gold/silver core/shell nanorod for detection of the ALP activity, accompanied by a rainbow-like multicolor change, and exhibiting a range of 5 – 100 mU mL^{-1} with a detection limit of 3.3 mU mL^{-1}, while the operation is relatively complex.[18] Therefore, developing a sensitive and stable strategy for ALP activity is anticipated urgently, which is significant and valuable for the clinical diagnosis and biomedical research.

Polypyridyl-based complexes can chelate with transitional metal ions, and have strong metal-to-ligand charge transfer (MLCT) absorption bands in the visible region.[19,20] Owing to the distinctive MLCT properties, the ligand of polypyridyl and its analogues have been widely used in practical applications such as detection of specific metal ions, while their transition metal complexes are used as colorimetric probes as well. For instance, ruthenium dyes, such as Ru(II) polypyridyl complexes, possess special optical properties; however, there are disadvantages of high cost and environmental toxicity.[21,22] In addition, complexes of bathophenanthroline and neocuproine with Fe^{3+} or Cu^{2+} have been widely used for colorimetric assays. Among them, copper(I) diimine complexes as a less-expensive, earth-abundant option were used widely.[23-25] Hu *et al.* use Cu(I)-BCDS complexes as the probes to constitute the colorimetric system.[26] By means of the property of MLCT, the ALP activity can be detected by the change of absorption. The LOD of ALP is 1.27 mU mL^{-1} and the dynamic range is 0 – 200 mU mL^{-1}.[27] This method is simple and efficient with high selectivity and interference immunity. Hence, the MLCT-based method of ALP activity has great potential and wide application to study. In addition, density functional theory (DFT) as a computational quantum mechanical modeling method can be used to investigate the electronic structure of complexes. Using this theory, the structure and properties of compounds can be estimated before any experiment, and it is beneficial for the progress of experiments and can make results manageable. Moreover, further time-dependent DFT (TD-DFT) can extend the basic ideas of ground-state DFT to extract the features of complexes like excitation energies, oscillator strengths and general time-dependent phenomena.[28] The ground-state geometries, electronic structure and electronic absorption spectra of complexes can be investigated using DFT and TD-DFT.

Cu(I) complexes of bicinchoninic acid (BCA) are frequently used as chromogenic indicators for the quantitative detection of proteins.[29,30] An operationally convenient visual detection of ALP activity was designed based on the complex of $Cu(BCA)_2^+$. The method anticipates that the L-ascorbic acid 2-phosphate (AAP) hydrolyze under the catalysis of ALP, and produce ascorbic acid (AA); then, Cu^{2+} is reduced to Cu^+ by AA; the BCA chelate with Cu^+ forming a purple-color complex as chromogenic probe, which finally results in an obvious color change of solution. The ALP activity could be quantitative determined by UV-vis absorption spectrometers and by naked-eye for qualitative analysis. Furthermore, it also works well in a complex biological solution, and is capable of directly detecting ALP activity in undiluted serum samples. This novel method provides a high selectivity and sensitivity route for the ALP detection in clinical diagnosis.

Experimental

Reagents and chemicals

Alkaline phosphatase (ALP, EC 3.1.3.1), L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (AAP), copper(II) sulfate pentahydrate, human serum albumin (HSA), trypsin, and hemoglobin were purchased from Sigma-Aldrich (St. Louis, MO). Acid phosphatase (ACP), bicinchoninic acid disodium salt hydrate (BCA), and 2-(ethylamino)ethanol (EAE) were obtained from TCI (shanghai) Development Co., Ltd. (Shanghai, China). Thrombin was purchased from Bersee Technology Co., Ltd. (Beijing, China). Sodium orthovanadate (Na_3VO_4) was obtained from Aladdin Industrial Inc. (Shanghai, China). Besides, *p*-nitrophenylphosphate (*p*NPP) was purchased from J&K Chemical Ltd. (Shanghai, China). All of the solutions were prepared with ultrapure water obtained from a Millipore Milli-Q water purification system (≥18.25 MΩ).

In this experiment, EAE–HCl buffer (0.05 M EAE, pH 9.8) was used as the reaction buffer throughout this study. Cupric salt ($CuSO_4$), sodium ascorbate, L-ascorbic acid-2-phosphate, and other solutions were separately dissolved in ultrapure water.

Apparatus

Cyclic voltammetry (CV) was carried out on a CHI 760D electrochemical workstation (Shanghai, China), while a 0.5 M KNO_3 solution was used as a supporting electrolyte, a glass carbon electrode (GCE, ϕ = 3 mm) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the counter electrode. UV-vis absorption spectra were recorded using a UV-3600 spectrophotometer (Shimadzu, Japan), 1 cm path length micro quartz cuvettes were used for detection.

Visual detection of the ALP activity

In a typical assay, 20 μL of 0.3 M AAP, 10 μL 20 mM $CuSO_4$, 20 μL 60 mM BCA, different concentrations of ALP and 50 mM EAE–HCl buffer in a total volume of 600 μL were mixed sufficiently. The mixture was incubated for 15 min at room temperature. The mixture solution was immediately transferred into a micro quartz cuvette to detect any increase in the absorbance at 562 nm using UV-vis spectroscopy. In addition, we also investigated the selectively of ALP in this assay for the complex environment in serum; several proteins were used as a control experiment. Finally, the ALP activity was detected directly by using serum samples in this assay, to evaluate the effectiveness and practicability of this method.

Inhibition efficiency of sodium orthovanadate

First, 20 μL of 0.3 M AAP, 10 μL of 20 mM $CuSO_4$, 20 μL of 60 mM BCA, different concentration of Na_3VO_4 and 50 mM EAE–HCl buffer were mixed in a 1-mL centrifugal tube in a total volume of 600 μL; then, 200 mU mL^{-1} ALP was added into the samples. The absorption spectra were measured after incubating for 15 min at room temperature.

The inhibition efficiency (IE) was calculate as:

$$IE = (A_1 - A_i)/(A_1 - A_0) \times 100\%.$$

A_1 stands for the absorbance of a sample with ALP but no inhibitor; A_i stands for the absorbance of samples with different concentrations of the inhibitor; A_0 stands for the absorbance of a sample without ALP or inhibitor.

Results and Discussion

Principle of the visual detection assay for ALP activity

The principle of visual detection for the detection of ALP is shown in Fig. 1. The catalytic property of ALP can facilitate the hydrolysis of AAP to produce ascorbic acid (AA); the Cu^{2+} will

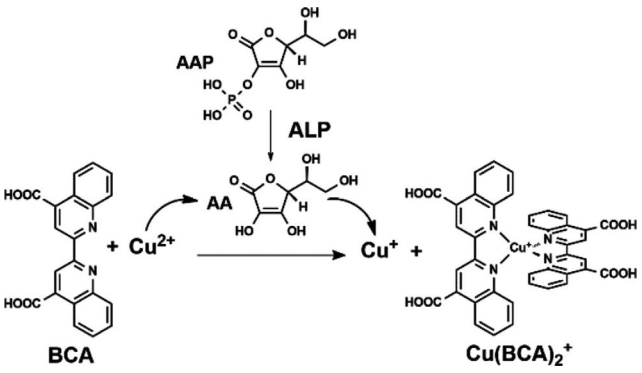

Fig. 1 Scheme of the ALP assay principle.

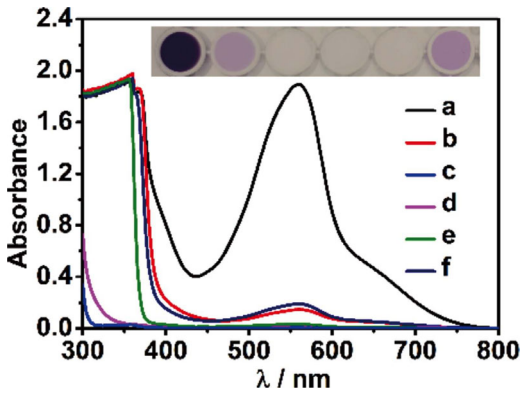

Fig. 2 Feasibility study. Absorption spectra of a solution prepared by adding all components and effects of different component absence: no absence (a); AAP (b); BCA (c); BCA and Cu^{2+}(d); Cu^{2+}(e); ALP (f). The inset shows the corresponding color photographs.

be reduced to Cu^{+} by AA; it then conjugates with BCA and form a water-soluble complex of Cu $(BCA)_2^{+}$ as a chromogenic probe; finally, it displays as purple color, while Cu^{2+} cannot. The probe has a featured intense absorption band at 562 nm in the UV-vis absorption spectrum, whose molar extinction coefficient is 7700 cm^{-1} M^{-1}.[31] On the contrary, in the absence of alkaline phosphatase, AA could not be produced, and a solution containing Cu^{2+} and BCA was colorless. The color of the solution is related to the ALP activity, this facilitated readout with the naked eye, which was used to construct a colorimetric sensor for ALP activity.

Feasibility of the visual detection assay for ALP activity

The feasibility of using Cu $(BCA)_2^{+}$ as a colorimetric probe for the ALP assay was demonstrated. As shown in curve a (Fig. 2), an obvious absorption band appears at 562 nm when ALP, AAP, Cu^{2+} and BCA were all added to the reaction solution, while there is a weak absorption at 562 nm and a small change of solution color if ALP, AAP, BCA, Cu^{2+} or Cu^{2+}/BCA was absent. Moreover, there is an absorption band at around 370 nm in the presence of BCA and through the experiment we found that the absorbance at 370 nm is proportional to the concentration of BCA ligand. Thus, the absorption band at 370 nm is the characteristic absorption of BCA ligand. In addition, the solution turned into purple only when ALP, AAP, Cu^{2+} and BCA were all added. These results clearly demonstrate that this method is valid and feasible to detect ALP activity. Due to the catalytic activity of ALP, AA is formed by hydrolysis, which reduces the Cu^{2+} to Cu^{+}. Thus, Cu^{+} combine with BCA as the chromogenic probe, which can reflect the ALP levels. As a result, the solution turned to a purple color vividly. Therefore, ALP activity could be directly determined with the naked eye simply through the color change phenomenon.

Electrochemical analysis and DFT calculation

Regardless of the absence and presence of BCA ligand in the solution, Cu^{2+} will be reduced by AA. We conducted cyclic voltammetry (CV) to examine the redox behavior of $Cu^{2+/+}$ redox couple on the basis of BCA ligand effect. As shown in Fig. S1 (Supporting Information), the redox property of the copper in a complex of $Cu(BCA)_2^{+}$ is different from its free state, whose stability constant is $10^{17.2}$.[32] The apparent formal potential ($E^{\circ\prime}$), as estimated from $E_{1/2} = (E_{red} + E_{ox})/2$,[33] of the $Cu^{2+/+}$ redox couple is about –0.035 V (*vs.* saturated calomel electrode (SCE)) when BCA is absent. However, in the presence of BCA ligand, the apparent formal potential shifts to a higher potential around 0.025 V (*vs.* SCE).[34] The increased $E^{\circ\prime}$ reveals that the Cu^{2+} is more difficult to be reduced in the presence of BCA. Due to the $3d^{10}$ electronic configuration of Cu^{+} center, while BCA is a strong field π acceptor ligand,[35] the delocalization electron from the *d*-orbitals of the metal center move to the π*-orbitals of the BCA ligand. The Cu^{2+} have a strong tendency to reduce to the Cu^{+} and produce the stable anion $Cu(BCA)_2^{+}$ in the presence of BCA, thereby raising its oxidation potential obviously. As a result, the complex of $Cu(BCA)^{+}$ is more stable than the Cu(II) complex, which provides a guarantee for the stability of the method.

Density function theory (DFT) calculations were performed to explore the ground-state molecular structure of $Cu(BCA)_2^{+}$, using the ADF2012.01 suit of program.[36,37] The ground state of Cu^{+} has a d^{10} electron configuration with a slightly distorted tetrahedral geometry (C_2).[38] The Cu^{+} center is in a four-coordinate tetrahedral geometry with two BCA ligands forming the complex of $Cu(BCA)_2^{+}$. The electron cloud distribution and flattening angle between ligands and the coordinating environment of the Cu metal center relate to the state of complex.[20] According to the results of the DFT calculation (Tables S1 and S2, Fig. S2 (Supporting Information)), when in the ground state, the biquinoline and biquinoline flattening angle is roughly 90 degrees. The computed HOMO energy is –4.803 eV and the LUMO energy is –2.274 eV. Thus, the estimated HOMO–LUMO gap is 2.53 eV. In HOMO-1, the electron density mainly located near the Cu^{+} center with a 64% contribution while electron shift to the BCA ligands upon excitation in LUMO+1 and the contribution of Cu is only 7%. This Franck–Cordon MLCT excited state with a $3d^{9}$ Cu^{2+*} center leads to a dramatic in solution color.[39] There are two absorption bands at 339 and 606 nm in visible region from 300 to 800 nm in the energy range investigated (Fig. S3, Supporting Information). The former peak, according to the experimental value at around 370 nm, reveals to the ligand character absorption (mainly from HOMO-3 to LUMO+3 and HOMO-2 to LUMO+2). The latter peak closed to the experiment value (562 nm), which assigned to the MLCT transition (mainly from HOMO-1 to LUMO+1).[40] The experiment measurements are in agreement with the calculation results, which achieved the prospective tentative of the assay for ALP activity.

Determination of ALP activity

The absorption spectra of different concentrations of ALP are shown in Fig. 3(A). As a result, an absorbance band at 562 nm was enhanced with the increase of ALP concentration.

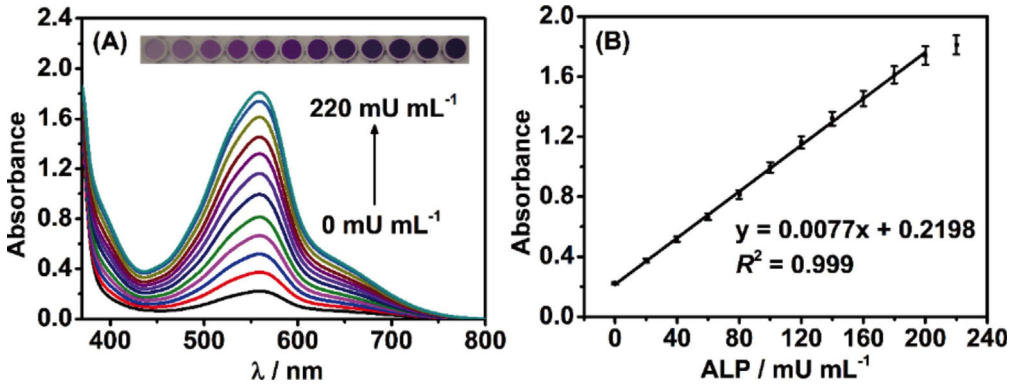

Fig. 3 (A) Changes in the absorption spectra of Cu(I)-BCA with different concentrations of ALP (0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 mU mL^{-1}) added. The inset shows the colorimetric responses in the presence of different concentration of ALP. (B) Linear relationship between the absorbance at 562 nm and the ALP concentration.

Table 1 Overview of fluorometric methods for the determination of tyrosinase or acid phosphatase

Method applied	LOD/U L^{-1}	Range/U L^{-1}	Reference
Fluorescence	0.12	0 - 6	F. Wang *et al.* (2014)
Fluorescence	10	100 - 800	L. Jia *et al.* (2010)
Electrochemical	0.1	0 - 10	J. Dong *et al.* (2014)
Colorimetric	0.1	0.5 - 10	J. Zhang *et al.* (2017)
Colorimetric	1.25	20 - 200	This work

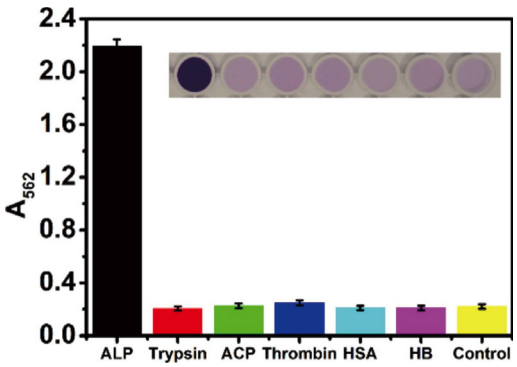

Fig. 4 Selectivity study. Changes in the absorption intensity of Cu(I)-BCA at 562 nm with the addition of ALP and other proteins. The inset shows the corresponding color photographs. ALP, 0.05 mg mL^{-1}; other proteins, 0.5 mg mL^{-1}. Control: the blank sample without ALP or other proteins.

The fast response of chromogenic guaranteed the high efficiency of the ALP activity detection. The experimental phenomena were observed that the ALP concentration addition provided an obvious color change of solution from mauve to modena. The color of final reaction solution is stable with little color change after keeping at 4°C for at least 24 h, which confirmed the stabilization of Cu^{+} with the BCA ligand.[41] The linear relationship between the absorbance and the ALP concentration was obtained (Fig. 3(B)): $y = 0.0077x + 0.2198$, where y stands for the absorbance intensity at 562 nm, x stands for the concentration of ALP in mU mL^{-1}. A good linear relationship ($R^2 = 0.999$) between absorbance and the ALP concentration is obtained in the wide range of 20 to 200 mU mL^{-1}. The limit of detection (LOD) at a signal-to-noise ratio of 3 is calculated to be 1.25 mU mL^{-1} (Table 1). This method is quite sensitive and convenient, so that the activity of ALP can be directly determined *via* naked eyes for qualitative analysis and use colorimetric analysis method for quantitative detection. Moreover, this method can practically detect the activity of ALP in human serums with high sensitivity while the normal range of ALP in health adult serums is 40 - 190 mU mL^{-1}.

Selectivity of visual detection assay for ALP

Various proteins in human blood may interfere with the results so that it is necessary to evaluate the selectivity of ALP in this method. We have made a comparative test of several common proteins (trypsin, thrombin, BSA, ACP and hemoglobin) to carry out a comparative experiment. No protein was added as a control group. The results are shown in Fig. 4, except for ALP; none of these proteins give a significant absorbance increase at 562 nm, and have no obvious color change that can be observed from inset. The experiment results show that the system for the determination of ALP has high selectivity. This method can thus be applied to directly detect the serum ALP levels, and could work well even in a complex biological environment.

Inhibitor screening

We also investigated the inhibition effect of the ALP inhibitor.[42] Sodium orthovanadate (Na_3VO_4), a frequently used inhibitor of ALP, was used to study the inhibition effect,[43] by changing the concentration of the inhibitor from 0 to 2.5 mM with 200 mU mL^{-1} ALP and keeping all other situations the same. The absorption spectrum of each group is presented in Fig. 5(A). The absorption intensity at 562 nm gradually decreased with the increase of the concentration of the inhibitor, which means that the Na_3VO_4 reduces the activity of ALP. These data were compared with previous experiment to calculate the inhibition efficiency. The IC_{50} value was calculated to be about 0.433 mM (Fig. 5(B)). As a competitive and reversible inhibitor of ALP, Na_3VO_4 can be used in the clinical treatment and other aspects. The color of the solution after the reaction becomes lighter with the increasing the inhibitor concentration. The results also demonstrate that the assay can be used for the facile screening of ALP inhibitors.[44]

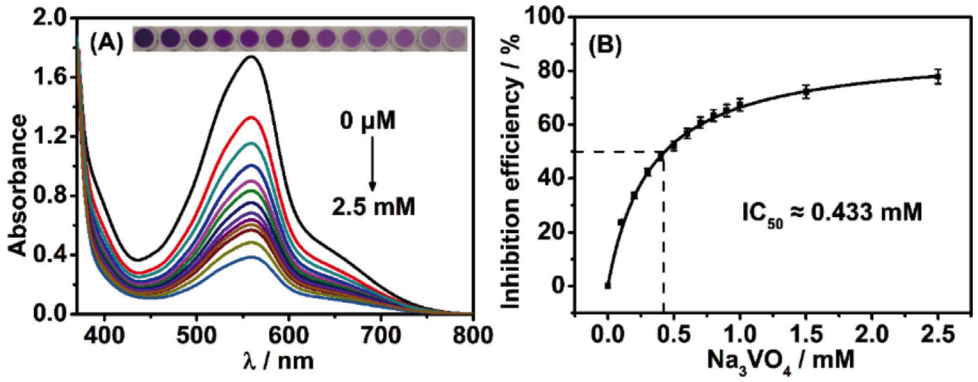

Fig. 5 (A) Changes in absorption spectra of Cu(I)-BCA with different concentration of Na_3VO_4 (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.5 mM) in the presence of 20 μL of 200 mU mL^{-1} ALP. The inset shows the corresponding color photographs. (B) Fitting curve relationship between the inhibition efficiency and Na_3VO_4 concentration.

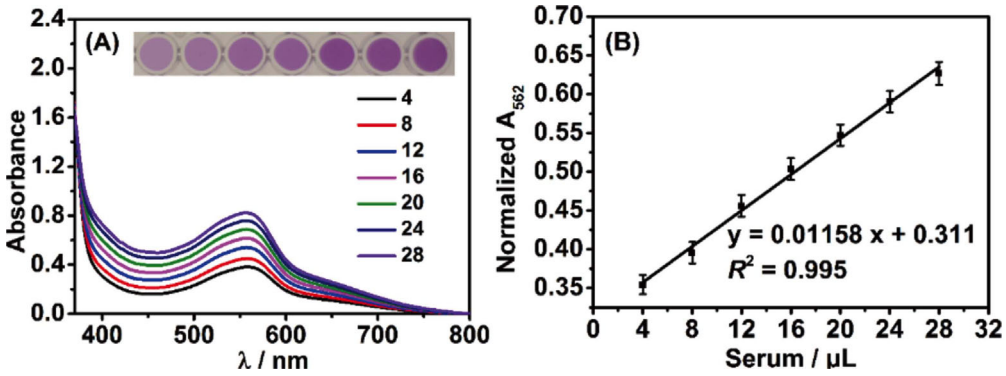

Fig. 6 (A) Changes in absorption intensity of solution toward different amount of serum (4, 8, 12, 16, 20, 24, 28 μL). The inset shows the corresponding color photographs. (B) Linear relationship between A_{562} and amount of serum.

Serum ALP levels detection

The assay was tested in human serum samples, which were obtained from fresh human blood by centrifuging. Instead of serum, plasma can be used for the detection of ALP activity as well. One aspect to note is the type of anticoagulant used in the preparation of plasma, only heparin sodium can be used because other anticoagulant can inhibit the activity of ALP.[45] Firstly, detect the absorption intensity of serum, itself, in a buffer as a baseline. Different amounts of serum to simulate different ALP levels for detection (Fig. 6). By calculating the net intensity absorbance of serum and comparing with the linear equation. We get the result that the sample serum ALP activity is 42.49 mU mL^{-1}. In order to demonstrate the interference immunity of the method, it is also needed to eliminate the colorimetric response of reducing interferents in the serum samples. The normal range of AA, cysteine (Cys), dopamine (DA), glucose (Glu), glutathione (GSH), urea in human serum are 34 – 114 μM,[46] 5.0 – 15 μM,[47] 0 – 888 pM,[48] 3.6 – 6.1 mM,[49] 0.26 – 0.34 mg mL^{-1},[50] and 2.0 – 7.1 mM,[51] respectively. In our experiment, the concentration of AA, Cys, DA, Glu, GSH, urea was chosen as 120 μM, 15 μM, 1.0 nM, 6.5 mM, 0.4 mg mL^{-1}, and 7.5 mM, respectively. As shown in Fig. S4 (Supporting Information), the reducing interferents in physiological levels, even undiluted serum samples (in the absence of AAP), has little response on the A_{562}. Therefore, the turn-on signal is contributed from the endogenous ALP of human serum samples, which demonstrates this method has strong anti-interference properties. Besides, the human blood (mainly from hemoglobin) has an obvious absorption band at around 410 nm, which overlaps with that of chromogenic product PNP of *p*NPP-based method, and a negligible absorption at 562 nm (Fig. S4). As a consequence, this method is practical, which can be used to directly determine the serum ALP levels by chromogenic analysis with high accuracy and does not produce false positive results.

On the basis of the results described above, it can be concluded that the method for detecting ALP activity has high practicability, stability and reproducibility. So this visual detection assay for ALP has great potential in the field of disease diagnosis and prognosis. The method overcomes some disadvantages of other assays. There are several advantages of this method compared with *p*NPP-based method. When the serum samples were detected, the existence of residual hemoglobin and bilirubin affect the veracity measurement, leading to false-positive results. Besides, the substrate *p*NPP is sensitive to the light and hydrolyze readily because of its poor stability. It is more practical because of the reliability and anti-interference of this visual detection so that avoiding the interference of hemoglobin and bilirubin in the whole blood.[52,53] Furthermore, this method is convenient and efficient in terms of the direct synthesis of a chromogenic probe by a two-step reaction. The reagents used in this system are commercially available and relatively inexpensive. By changing the colorimetric probe to other

analogous copper diamine complexes, this method has a more extensive research prospect and room for improvement. Taking these results into account, the method of $Cu(BCA)_2^+$ as a chromogenic probe for ALP activity in serum has a high application value in clinical detection and analysis of diseases.

Conclusions

In summary, we have developed a novel visual detection assay for ALP detection on the basis of the distinctive MLCT properties of a chromogenic probe. Needless of any complex reaction process and reaction conditions, ALP can be detected visually with high sensitivity through the color change with naked eyes or an accurate spectrophotometer. Moreover, the analysis performance of the visual detection method is practically with the range of detection being is 0 – 200 mU mL^{-1} which is suitable to human blood levels. The results of detecting the serum ALP levels showed that this system has high application value in clinical detection and the analysis of diseases. The BCA ligand of the method is replaceable by its analogues to improve the performance in order to meet the higher requirements in medicine and physiology. This assay provides a kind of detection method for other enzymes even in the field of microbial detection, and furthermore advances the molecular tool kit of ALP, facilitating the investigation of this biological and clinical roles in living systems.

Acknowledgements

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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