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Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.8b01371 • Publication Date (Web): 23 Apr 2018

Alkaline Phosphatase Assay Based on the Chromogenic Interaction of Diethanolamine with 4-Aminophenol

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ABSTRACT: Diethanolamine (DEA) has been extensively utilized as an alkaline buffer in current assays of alkaline phosphatase (ALP) activity in the past decades. While playing the role of a buffer, DEA has been widely ignored about its chemical reactivity in such assays. Herein, we report an interesting chromogenic interaction between DEA and 4-aminophenol (AP) in the presence of H_2O for the first time, which inspires us to develop a novel DEA-participated ALP activity assay by using 4-aminophenyl phosphate (APP) as a substrate. This APP/DEA-based colorimetric approach has been proved to be comparable and even superior to the conventional *p*-nitrophenyl phosphate (pNPP)-based one, especially in the low ALP activity region due to its higher sensitivity. The clear response mechanism and excellent sensing performance ensure that it can be further applied in determining ALP activity in real biological samples, screening potential ALP inhibitors in vitro, establishing ALP-enabled ELISA and even fluorophore-assisted fluorescent ALP activity assay. It is demonstrated that this strategy not only possesses a good feasibility but also exhibits a promising outlook for a series of ALP-related and -extended detections.

The ubiquitous alkaline phosphatase (ALP, EC 3.1.3.1) is a homodimeric digestive enzyme that is involved in phosphate metabolism and widely distributed in various biological organisms from bacteria to mammalian.[1-3] ALP exhibits an optimal enzyme reaction rate in an alkaline environment and can specifically catalyze the hydrolysis of phosphate ester groups. Owing to its widespread prevalence, the level of serum ALP serves as a significant indicator of certain diseases, such as rickets, osteomalacia, hepatitis, periodontal diseases and even prostatic cancer et al.[4,5] Moreover, ALP is frequently employed as a signal enzyme in the enzyme-linked immunosorbent assay (ELISA), dairy industry and so on.[6-8] Therefore, developing a remarkable and convenient assay for ALP activity is necessary and important to the related disease diagnosis, drug screening and other extended applications.[9-11]

It is particularly noteworthy that ALP has broad substrate specificity and ability to dephosphorylate or transphosphorylate a large number of phosphated molecules.[12] On the basis of the clearly catalytic principle and enough desirable substrates, various analytical methods including electroanalytical, fluorometric and colorimetric assays have been developed for determining ALP activity.[13-15] In this regard, the phenyl phosphate compounds have been used in the colorimetric determination of ALP activity for more than seventy decades.[16,17] Especially, *p*-nitrophenyl phosphate (pNPP) is one of the most understood and commercially available chromogenic substrates in the conventional colorimetric assay for ALP activity, in which pNPP can be converted into a colored product, p-nitrophenol (NP).[16,18] Moreover, 4-methylumbelliferyl phosphate and several other phosphate functionalized molecules have been already developed and used as fluorogenic substrates.[19] With rapid development of plasmonic and fluorescent nanomaterials, a series of booming colorimetric and fluorimetric ALP assays have been also established based on the differential influence of certain substrates and corresponding products on the property of such nanomaterials.[9,10,14]

Generally, higher enzyme reaction rate of ALP can be observed in alkaline solution (typical pH = 9.8), therefore, a common buffer reagent, diethanolamine (DEA), is employed as the main component in most current assays of ALP activity.[20,21] In fact, DEA is prone to provide a proper buffer pH for ALP detection while it does not influence the enzymatic reaction and signal generation owing to the favorable chemical stability and biocompatibility.[22] Thus, it is relatively scarce to pay attention to the DEA molecule and there is no report concerning the chemical reactivity of DEA except for the pH-buffering effect in ALP assay to the best of our knowledge.

As a typical and desired substrate, 4-aminophenyl phosphate (APP) can be hydrolyzed and transformed into 4-aminophenol (AP) in the presence of ALP.[23,24] In comparison with APP, the enzymatic product AP exhibits better electrochemical activity and can be oxidized more easily.[25] Therefore, both APP-based electrochemical ALP activity assay and resultant ALP-enabled electrochemical immunoassay have been proposed using redox cycling by a reducing agent.[26-28] Furthermore, Lin et al. have developed an ALP-triggered colorimetric ELISA based on the fact that ALP could catalyze the hydrolysis of APP into AP, which could reduce silver ions to generate silver shell onto the

gold nanobipyramids (Au NBPs) and thus resulted in a significant impact on the localized surface plasmon resonance properties of Au NBPs.[29] On the other hand, however, it is very limited in the direct colorimetric assays for ALP by using APP as the substrate, due to the indistinguishable differences in the colors and absorption spectra between the colorless APP and AP aqueous solutions, unlike traditional pNPP/NP substrate/product pair.[20]

For the first time, however, we discovered a very interesting phenomenon that the absorption spectrum of AP in DEA buffer solution had a new absorption peak centered at about 365 nm after incubation, which was not obtained if using APP instead of AP. We then tried to explain this phenomenon by demonstrating the mechanism and behavior of the chromogenic interaction between DEA and AP. Inspired by such discriminability and difference between APP and AP, we report here an original colorimetric assay for ALP activity by employing APP as the substrate, in which DEA is not only a pH-buffering agent, but also a reactant in the aforementioned chromogenic interaction. More significantly, such facile and exclusive interaction between DEA and AP demonstrated high sensitivity, good selectivity, and universal applicability in ALP assay. According to the clear response mechanism and excellent sensing performance, such APP/DEA-based ALP assay is extended into determining ALP activity in real biological samples, screening potential ALP inhibitors and establishing ALP-enabled ELISA. In addition, an original fluorescent ALP assay is also rationally developed through the combination of the proper fluorophore (silver/gold bimetallic nanoclusters capped with 11-MUA, AgAuNCs@MUA as an example).

EXPERIMENTAL SECTION

Reagents and Apparatus. Diethanolamine (DEA), 4-aminophenol (AP), 4-aminophenyl phosphate monosodium salt hydrate (APP), 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP), silver nitrate ($AgNO_3$), alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa, 11-mercaptoundecanoic acid (MUA), hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO). Magnesium chloride ($MgCl_2$), sodium molybdate dehydrate (Na_2MoO_4), trisodium tetraoxovanadate dodecahydrate (Na_3VO_4) and sodium hydroxide (NaOH) were purchased from Aladdin Industrial Corporation (Shanghai, China). Fetal bovine serum was purchased from SangonBiotech Co. Ltd. (Shanghai, China). The microplates coated with anti-human AFP, lyophilized AFP protein standard, AFP detection antibody, sample diluent buffer and wash buffer were purchased from RayBiotech (Norcross, GA). ALP-conjugated secondary antibody was purchased from Abcam (Cambridge, MA). Ultrapure water (18.2 MΩ cm) was used in all aqueous solution.

Absorption and fluorescence spectra were recorded on CARY 500 UV−Vis-NIR Varian spectrophotometer (CA) and Hitachi F-4600 spectrofluorometer (Tokyo, Japan), respectively, by using a 1 cm path length quartz cell at room temperature. Transmission electron microscopy (TEM) images were acquired by a FEI Tecnai G2 F20 S-TWIN (OR).

The Interaction Between DEA and AP. The 400 mM DEA stock solution were prepared by dissolving DEA into the ultrapure water and adjusting pH values to 9.8. The interaction between DEA and AP was performed by adding different amounts of DEA stock solution into freshly prepared AP aqueous solutions. The absorption spectrum measurements were carried out after incubation at 37 °C for 60 min.

APP/DEA-Based Assay for ALP Activity. The ALP activity assay was performed with the following procedures. Volumes of the 400 μL of APP (2 mM), 400 μL of DEA (400 mM, pH 9.8), 80 μL of $MgCl_2$ (1 mM) and 320 μL of water were injected into a 2.0 mL microcentrifugal tube. Next, the 400 μL of freshly prepared ALP standard aqueous solutions with different activities ranging from 0 to 200 mU/mL were added into the solutions, respectively. The above mixture solutions were incubated at 37 °C for 60 min before the absorption spectrum measurements. The APP-based and the control pNPP-based assays for ALP activity in diluted fetal bovine serum sample (FBS, 2%) were carried out under the same conditions.

ALP Inhibition Assay. Volumes of the 400 μL of APP (2 mM), 400 μL of DEA (400 mM, pH 9.8), 80 μL of $MgCl_2$ (1 mM), and 80 μL of freshly prepared ALP standards (100 mU/mL) and 240 μL of water were injected into a 2.0 mL microcentrifugal tube. Then the 400 μL of Na_3VO_4 or Na_2MoO_4 with various concentrations were added into the solutions, respectively. The above mixture solutions were incubated at 37 °C for 60 min before the absorption spectrum measurements.

APP-Based ELISA for Human AFP. First, volumes of the 100 μL of AFP standards (0 to 100 ng/mL) were added into the microplates coated with anti-human AFP and the microplates were incubated overnight at 4°C. After incubation, the removal of AFP solutions and washing processes were executed sequentially. Next, 100 μL of 500-fold diluted AFP detection antibody was added, followed by incubation at room temperature for 60 min with gentle shaking. After removal and washing again, 100 μL of 3000-fold diluted ALP-conjugated secondary antibody was added, followed by incubation for 60 min with gentle shaking. After removal and washing processes, the 62.5 μL of APP (2 mM), 62.5 μL of DEA (400 mM, pH 9.8), 12.5 μL of $MgCl_2$ (1 mM) and 112.5 μL of water were added. The absorption spectrum measurements were carried out after gentle shaking at 37 °C for 60 min.

AgAuNCs@MUA-Assisted Fluorescent Assay for ALP Activity. The silver/gold bimetallic nanoclusters capped with 11-MUA (AgAuNCs@MUA) were synthesized and purified according to a modified strategy reported previously.[30,31] In brief, MUA (13.1 mg) and NaOH (6.0 mg) were dissolved in water (8.5 mL), followed by addition of the solution of $AgNO_3$ (0.5 mL, 10 mM) and $HAuCl_4$ (1.0 mL, 10 mM) at room temperature. The thorough mixture was left to stand for 5 h at room temperature, and then was dialyzed with an 8−14 kDa cutoff dialysis bag, against ultrapure water for more than 24 h with four changes.

The fluorescent ALP activity assay was performed using the following procedures. Volumes of the 200 μL of APP (2 mM), 200 μL of DEA (400 mM, pH 9.8), 40 μL of $MgCl_2$ (1 mM) and 160 μL of water were injected into a 2.0 mL microcentrifugal tube. Next, the 200 μL of freshly prepared ALP standard aqueous solutions with different activities ranging from 0 to 80 mU/mL were added into the above solutions, respectively. After gentle shaking at 37 °C for 60 min, 800 μL of 100-fold diluted AgAuNCs@MUA was added, followed by the fluorescence spectrum measurements.

RESULTS AND DISCUSSION

The Chromogenic Interaction Between DEA and AP. As a typical phosphated molecule, APP is frequently employed as the substrate in ALP activity assays, in which the oxidation-reduction properties between APP and the corresponding enzymolysis product AP can be differentiated by electrochemical measurements.[25-27] Besides the better electrochemical activity, AP has no obviously differences in absorption or reactivity compared to APP in previous reports. On the other hand, owing to its buffer capacity in alkaline environment, the colorless DEA solution used to serve as a pH mediator in ALP activity assay, as well as in ALP-enabled ELISA.[20] In fact, the aqueous solution of sole AP or DEA kept colorless under the incubation at 37 °C for 60 min, where the representative absorption spectrum of AP exhibited an absorption peak around 305 nm and that of DEA lacked a characteristic peak (Figure 1). However, we were surprised that the mixture solution of AP and DEA displayed an apparent absorption peak centered at about 365 nm. Such an interesting phenomenon as well as the new characteristic peak has not been reported in the previous research as far as we know. Therefore, we further evaluated this unusual interaction between AP and DEA.

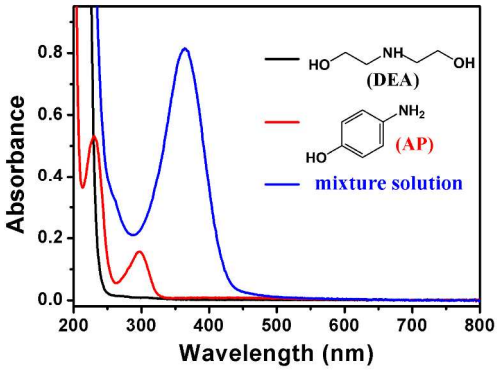

Figure 1. Absorption spectra of DEA (black), AP (red) and DEA + AP (blue) aqueous solution after incubation at 37 °C for 60 min.

First, we investigated the concentration effect of AP to the resultant mixture. As shown in Figure 2A and 2B, by adding different concentrations of AP into the aqueous solution of constant DEA (100 mM, pH 9.8), the absorption spectra of the mixture brought a continuous enhancement and the absorbance values at 365 nm presented a quasi-linear relationship as AP concentrations ranging from 0 to 200 μM. Second, the absorption spectra in Figure 2C demonstrated that pH values of the solution could influence the AP/DEA interaction and the optimum pH value was around 10 according to the maximum absorbance. This strong alkaline pH value was similar to that of DEA buffer, which was commonly used in the ALP activity assay. We next evaluated the concentration effect of DEA to the resultant mixture. By adding different concentrations of DEA (pH 9.8) into the aqueous solution of constant AP (100 μM), the corresponding absorption spectra in Figure 2D had a quick to slow absorption enhancement under increased concentrations (typically > 100 mM). Incubation time and temperature could also affect the interaction between AP and DEA. As presented in Figure 2E and 2F, the time-dependent absorption spectra of the mixture solutions and plots of absorbance values at 365 nm as a function of time showed that the AP/DEA interaction accelerated over the initial stage and then reached a plateau after 60 min. In addition, higher incubation temperature would be only beneficial to the rapid completion of the reaction under current conditions (data not shown).

Insight into the Interaction of DEA with AP in H_2O. The absorption peak at 365 nm was proposed to be related to the reaction between DEA and AP. From the viewpoint of synthetic organic chemistry, the synthesis and isolation of the corresponding product is impossible under our current conditions because the stoichiometry of DEA to AP is at least 200 equiv. excess and both of the substrates have to be under low concentrations for a reasonable rate of transformation. Nevertheless, we performed the reaction of DEA with AP under

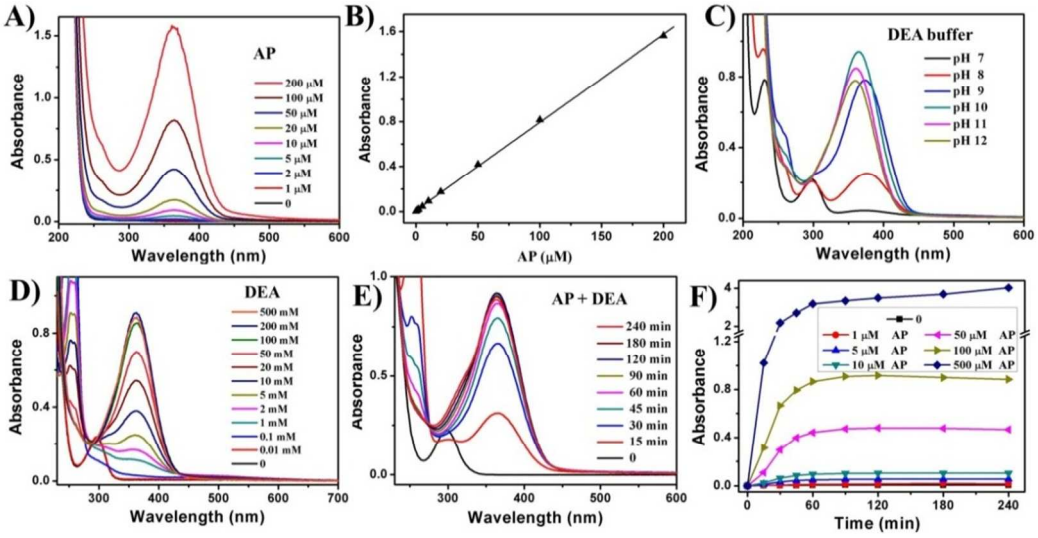

Figure 2. (A) Absorption spectra and (B) absorbance values at 365 nm of the solutions by adding different concentrations of AP (0−200 μM) into DEA (100 mM, pH 9.8) after incubation at 37 °C for 60 min. (C) Absorption spectra of the solutions by adding AP (100 μM) into DEA (100 mM) at different pH values from 7 to 12 after incubation at 37 °C for 60 min. (D) Absorption spectra of the solutions by adding AP (100 μM) into different concentrations of DEA (0−500 mM, pH 9.8) after incubation at 37 °C for 60 min. (E) The time-dependent absorption spectra of the mixture solutions of AP (100 μM) and DEA (100 mM, pH 9.8) at 37 °C. (F) Absorbance values at 365 nm of the solutions by adding different concentrations of AP (0−500 μM) into DEA (100 mM, pH 9.8) as a function of incubation time.

literature reported condition[32] and obtained a thermodynamically stable product, 1-(4-hydroxyphenyl)piperazine (HPPA) (Figure 3A). HPPA was then characterized under a series of solution conditions. As shown in Figure 3B, to avoid the influence from DEA and AP, HPPA was characterized immediately after dissolved in pure H_2O and ethyl acetate, respectively. Interestingly, no absorption peak at 365 nm appeared under both conditions (line a and c in Figure 3B). In sharp contrast, after incubation for 60 min, the corresponding absorption appeared in aqueous solution (line b) while be absent in ethyl acetate solution (data is very similar to line c and not shown), indicating the absorption peak at 365 nm is originated from the specific interaction between HPPA and H_2O. By extracting the resultant mixture of HPPA and H_2O with ethyl acetate, we found the absorption peak at 365 nm was maintained. It should be noted that the interaction between HPPA and H_2O is quite incomplete, with most of HPPA remained. Although the low conversion rate of HPPA in H_2O led to a difficulty in isolation and full characterization of the resultant, the high UV-absorbance-sensitivity and detectability of the resultant made it an observed advantage for potential ALP activity assay.

APP/DEA-Based Colorimetric Assay for ALP Activity. Under similar conditions, no chromogenic product with an absorption peak at 365 nm could be obtained when we used APP to incubate with DEA (Figure 4A). These results further demonstrated the high chemical selectivity of the interaction between AP and DEA. On the other hand, it is not surprising that the incubation of sole ALP in DEA aqueous solution could not induce any featured absorption. As expected, the resultant mixture exhibited an apparent absorption peak centered at 365 nm by simultaneously adding APP and ALP into DEA aqueous solution followed by incubating at 37 °C for 60 min. Inspired by such a phenomenon and the catalytic capability of ALP in the hydrolysis of APP to AP, we proposed an APP/DEA-based colorimetric assay for ALP activity by using APP as the substrate and the AP/DEA interaction as a signal generation unit. More significantly, the most commonly used buffer in the enzymatic reaction by ALP is just alkaline DEA buffer (pH = 9.8), which is also very beneficial to the subsequent chromogenic interaction between AP and DEA. Therefore, both the enzymatic and chromogenic processes of our ALP assay can be performed in one pot at the same time.

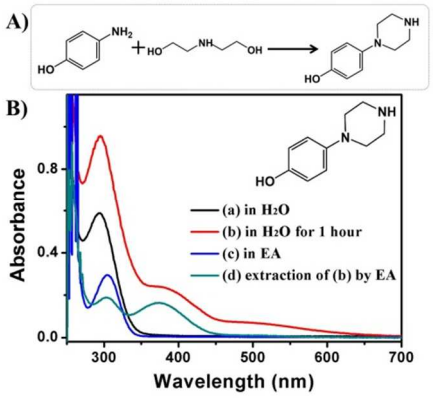

Figure 3. (A) Chemical structures and proposed reaction of AP, DEA and HPPA. (B) Absorption spectra of HPPA in aqueous solution (a), in aqueous solution after incubation for 60 min (b), in ethyl acetate solution after incubation for 60 min (c) and the ethyl acetate (EA) extraction from b (d), respectively.

In order to achieve the optimal performance of our proposed assay, several experimental conditions were investigated and optimized. Obviously, DEA was not only a buffer component but also served as a reactant in the chromogenic reaction. Thus the adopted concentration of DEA here was 100 mM, which was prone to provide a proper buffer pH and enough to react with the enzymatic product AP according the investigation on the AP/DEA interaction. It is suggested that magnesium ions (Mg^{2+}) act an important role in accelerating the enzymatic reaction of phosphatase.[33] Our results in Figure S1 showed that higher concentrations of Mg^{2+} could induce a more marvelous enhancement of the absorption at 365 nm. However, the absorbance values had little change when adding Mg^{2+} concentration more than 50 μM, and such concentration was chosen in the following assay experiments. In addition, the concentrations of APP also influenced the absorption spectrum in the assay process. As shown in Figure S2, under constant DEA and Mg^{2+} ions concentrations, the absorption values at 365 nm exhibited a gradual enhancement with an increased concentration of APP. Comprehensive considering the signal accuracy and reagents consumption, we selected APP (500 μM) in 100 mM DEA buffer (pH 9.8) containing Mg^{2+} (50 μM) as the standard sensing solution to determine the activity of ALP.

In the presence of serial diluted ALP standards, the aforementioned sensing solutions were incubated at 37 °C for 60 min and the absorption spectra recorded in Figure 4B showed that the absorbance at 365 nm of the mixture solution had a continuous enhancement with increasing ALP activities (0 – 50 mU/mL). In particular, a quasi-linear relation between absorbance values and ALP activities were presented between 0.01 to 10 mU/mL and the fitted data could be expressed as A_{365} = 0.0033 + 0.144 C_{ALP} (mU/mL), R^2= 0.995 (Figure 4C and 4D). Moreover, the inset graph of Figure 4C showed that 0.01 mU/mL of ALP could clearly result in an obvious increase in absorbance values and be easily identified by the absorption spectrophotometer.

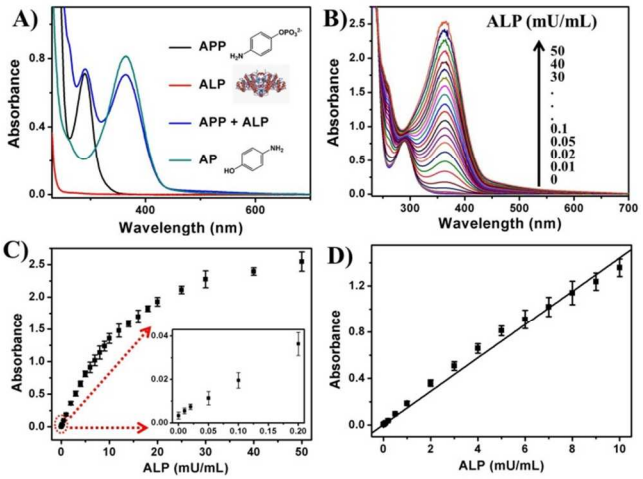

Figure 4. (A) Absorption spectra of the solutions by adding APP (black), ALP (red), APP + ALP (blue) and AP (green) into DEA after incubation at 37 °C for 60 min. (B) Absorption spectra, (C) absorbance values at 365 nm and (D) expanded linear response region of the sensing system towards ALP standards with various activities (0−50 mU/mL).

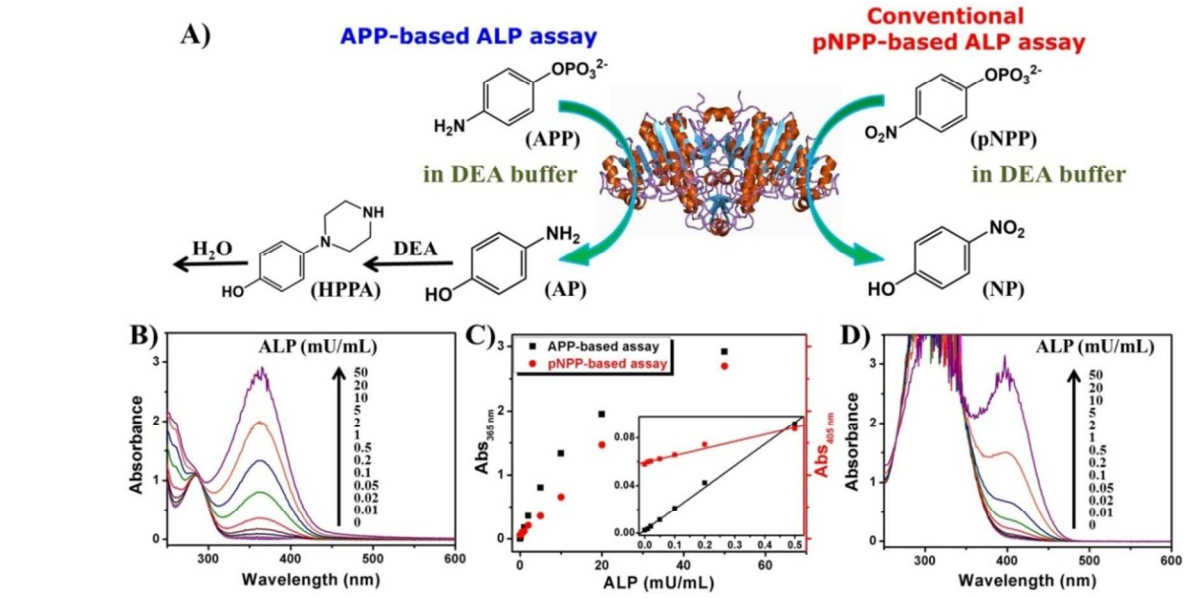

Figure 5. (A) Schematic representation of the APP-based and pNPP-based assays for ALP activity. Absorption spectra of the APP-based (B) and pNPP-based (D) sensing systems towards ALP standards with 2% fetal bovine serum. (C) Plots of absorbance values at 365 nm for APP-based assay (black) and at 405 nm for pNPP-based assay (red) as a function of ALP activities (0−50 mU/mL). Inset: expanded region.

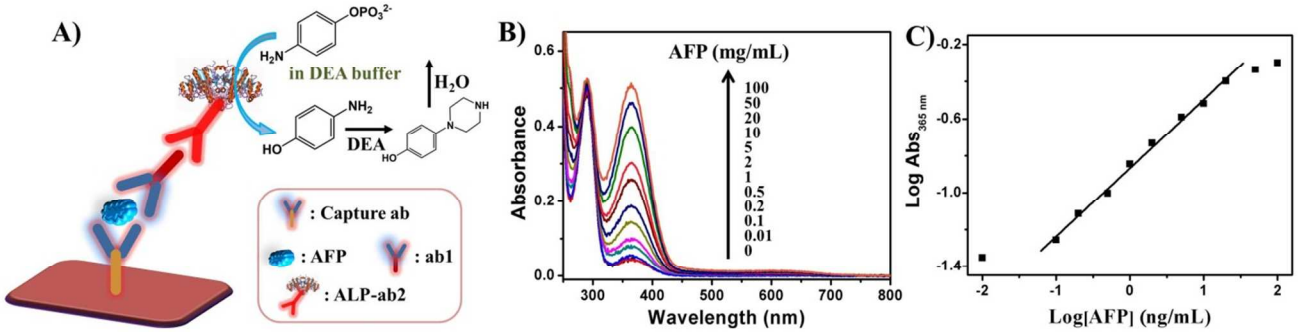

Figure 6. (A) Schematic representation of the immunoassay based on the APP-based ALP assay and conventional ELISA platform. (B) Absorption spectra and (C) absorbance values at 365 nm of the ELISA system toward AFP standards (0.01 – 100 ng/mL).

As described in Figure 5A, the conventional colorimetric assay for ALP activity usually employs a commercially available compound pNPP as the substrate and pNPP can be converted into a colored product NP in DEA buffer. Alternatively, our proposed ALP assay needs to add commercially available APP into DEA buffer. To evaluate the feasibility and potential bioapplication of our developed assay, we carried out the APP/DEA-based and conventional pNPP-based sensing systems, respectively, in determination of ALP activity in 2% fetal bovine serum (Figure 5). Absorption spectra reveal that both sensing systems are promising in real biological sample analysis based on the high specificity in terms of the phenolic phosphates. However, our developed APP/DEA-based assay is superior in the sensitivity to pNPP-based one, especially in low ALP activity region, due to its higher sensitivity in absorption with increasing ALP activities (Figure 5C). On the other hand, the different detection window (absorbance values at 365 nm for APP/DEA-based assay and at 405 nm for pNPP-based one) might make the APP/DEA-based assay as a substitute or supplement for the conventional pNPP-based one.

Additional Application of the ALP Activity Assay. To further confirm the availability of our developed APP/DEA-based ALP activity assay, we demonstrated the possibility of applying the assay for inhibitor screening and ALP-enabled immunoassay. First, considering the integral role in metabolism, it is necessary to develop a facile inhibitor screening method for ALP, where molybdate (MoO_4^{2-}) and orthovanadate (VO_4^{3-}) are commonly used as the model inhibitors in vitro.[34-36] By premixing increasing concentrations of MoO_4^{2-} or VO_4^{3-} with constant activity of ALP (5 mU/mL), as shown in Figure S3 and S4, the enzymatic activity were efficaciously inhibited and the absorption peak centered at 365 nm exhibited a gradual reduction. Plotting the absorbance values at 365 nm versus the logarithm of the MoO_4^{2-} or VO_4^{3-} concentration, a typical sigmoidal profile were obtained and the IC_{50} values (50% maximal inhibitory concentration) were calculated to be approximate 1.56 mM for MoO_4^{2-} and 63.3 μM for VO_4^{3-}. Both of the values were in the same order of magnitude as those previously reported by other assays for ALP activity.[34,35]

Next, being one of most frequently used enzymes in ELISA, ALP activity assay has usually been extended into the ALP-enabled immunoassay.[6] As descripted in Figure 6A, we used AFP as a model antigen target, combining with commercially available antibodies and antibody-labeled ALP to demonstrate the analytical behavior of ALP-labeled ELISA for a proof-of-concept.[37] Under the controls, the addition of individual anti-

human AFP (capture Ab), AFP standard and detection antibody (ab1), respectively, had no influence on absorption spectrum of the aforementioned ALP sensing solution (data not shown). Following the routine immobilization of the antigens and antibodies on a 96-well plate, our proposed sensing solution was introduced and the absorption spectra were recorded. The results in Figure 6 demonstrated that the absorption spectrum and absorbance values at 365 nm of the reaction solutions exhibited a gradual enhancement with the AFP concentrations increasing from 0 to 100 ng/mL. In particular, a quasi-linear relationship between double logarithmic absorbance values and AFP concentration could be obtained from 0.1 to 20 ng/mL and the fitted data could be expressed as Log (A_{365}) = – 0.867 + 0.370 Log (C_{ALP}) (ng/mL), R^2= 0.994. In such sensitive APP/DEA-based ELISA, 0.1 ng/mL of AFP could be easily identified by the absorption spectrophotometer and this value was lower than those in previously reported ELISA.[22,38]

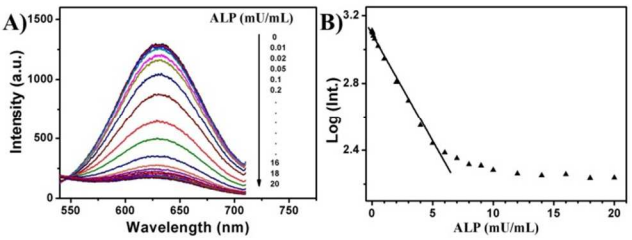

Figure 7. (A) Fluorescence spectra of the AgAuNCs@MUA-APP sensing system towards ALP standards with various activities (0−20 mU/mL). (B) Plots of the corresponding logarithm of fluorescence intensities at 630 nm as a function of ALP activities.

AgAuNCs@MUA-Assisted Fluorescent Assay for ALP Activity. Besides colorimetric method, the fluorescent assays have several advantages and are also well exploited due to their intrinsic high sensitivity and fast response time. Inspired by the fluorescence quenching phenomenon of some fluorophores with certain chromogenic reagents, a series of colorimetric responses has been further extended into fluorescent assays by means of proper fluorescent materials.[39-41] In fact, adding another signal sensing mode is conducive to providing more accurate and convincing detection results. On the basis of the unique optical properties and large stokes shift,[42,43] we introduced a type of promising fluorescent noble metal nanoclusters (e.g. silver/gold bimetallic nanoclusters capped with 11-MUA, AgAuNCs@MUA) into APP/DEA-based ALP activity assay.

As shown in Figure S5, the typical TEM image and fluorescence excitation/emission spectra confirmed the generation of AgAuNCs@MUA. More significantly, AgAuNCs@MUA exhibited an intense excitation band with a broad range around 350 nm, which was partially overlapped with the absorption spectrum of AP/DEA mixture. By adding AP-DEA mixture solution into AgAuNCs@MUA solution, the fluorescence emission peak around 630 nm of AgAuNCs@MUA was remained, while the fluorescence intensity exhibited a gradual decrease owing to the quenching effect of the resultant HPPA derivative (Figure S6). In addition, the presence of individual ALP, APP, AP and even strong alkaline DEA solution did not affect the fluorescence of AgAuNCs@MUA (data not shown). Therefore, it encouraged us to develop a fluorescent ALP activity assay by means of ALP-enabled generation of chromophore and its quenching effect on the fluorescence of AgAuNCs@MUA (Figure 7). The fluorescent sensing solution including APP, DEA and AgAuNCs@MUA clearly exhibited a characteristic emission of the AgAuNCs. With the activities of incoming ALP standards increasing from 0 to 20 mU/mL, the fluorescence emission intensity decreased gradually. Moreover, a quasi-linear relationship between the logarithm of the intensity values at 630 nm and ALP activities could be obtained from 0 to 5 mU/mL, in which the fitted data could be expressed as Log (I_{630}) = 3.091 – 0.370 C_{ALP} (mU/mL), R^2= 0.991. Such fluorescent sensing performance were similar to that in the colorimetric response, undoubtedly suggesting fluorophore-assisted fluorescent assay possessed considerable operability and making the detection results more convincing.

CONCLUSION

In summary, an interesting chromogenic interaction between DEA and AP has been discovered and studied in detail. The absorption spectrum of the AP and DEA in the presence of H_2O has a new absorption peak centered at about 365 nm, which is not presented if using APP instead of AP. Inspired by the ALP-triggered hydrolysis of APP into AP, a well-defined colorimetric assay for ALP activity has been rationally developed based on such discriminability between APP and AP. For the first time, DEA behaves as not only a buffer component but also a reactant in a chromogenic reaction for assaying ALP. By means of the clear response mechanism and excellent sensing performance, our developed DEA-participated ALP activity assay is successfully applied in measuring the real biological samples, screening potential inhibitor, constructing the ALP-enabled immunoassay and even fluorophore-assisted fluorescent assay. Thus, this promising assay will not only be useful in direct detection of ALP activity but also in other ALP-related detection and sensing systems. More significantly, the APP/DEA-based assay is superior to pNPP-based one in terms of the sensitivity. On the other hand, the different detection window might make such assay as a substitute or supplement for the conventional pNPP-based one.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Effect of Mg^{2+} and APP concentrations on the colorimetric ALP assay, detailed absorption spectra of the assay for inhibitor screening (MoO_4^{2-} and VO_4^{3-}), typical TEM image and fluorescence excitation/emission spectra of AgAuNCs@MUA and the effect of AP in DEA solution on the fluorescence of AgAuNCs@MUA (PDF)

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

Financial supports by the National Natural Science Foundation of China (Grant Nos. 21605139, 21435005, 21627808), the National Key Research and Development Program of China (2016YFA0201301) and Key Research Program of Frontier Sciences, CAS (QYZDY-SSW-SLH019).

REFERENCES

(1) Alonso, A.; Sasin, J.; Bottini, N.; Friedberg, I.; Friedberg, I.; Osterman, A.; Godzik, A.; Hunter, T.; Dixon, J.; Mustelin, T. Protein tyrosine phosphatases in the human genome. *Cell* **2004**, *117*, 699-711.

(2) Lalles, J. P. Intestinal alkaline phosphatase: novel functions and protective effects. *Nutr. Rev.* **2014**, *72*, 82-94.

(3) Harris, H. The human alkaline-phosphatases: what we know and what we don't know. *Clin. Chim. Acta* **1990**, *186*, 133-150.

(4) Bowers, G. N.; Mccomb, R. B. Measurement of total alkaline phosphatase activity in human serum. *Clin. Chem.* **1975**, *21*, 1988-1995.

(5) Rao, G. M. M.; Morghom, L. O. Correlation between serum alkaline phosphatase activity and blood glucose levels. *Enzyme* **1986**, *35*, 57-59.

(6) de la Rica, R.; Stevens, M. M. Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye. *Nat. Nanotechnology* **2012**, *7*, 821-824.

(7) Wang, Y.; Yang, J. Y.; Xu, Z. L.; Qi, P.; Shen, Y. D. Synthesis of novel hapten and development of monoclonal antibody-based enzyme-linked immunosorbent assay for malachite green in fish samples. *Chin. J. Anal. Chem.* **2016**, *44*, 1385-1393.

(8) Hoy, W. A.; Neave, F. K. The phosphatase test for efficient pasteurisation. *Lancet* **1937**, *2*, 595-598.

(9) de la Rica, R.; Stevens, M. M. Plasmonic ELISA for the detection of analytes at ultralow concentrations with the naked eye. *Nat. Protoc.* **2013**, *8*, 1759-1764.

(10) Xianyu, Y. L.; Wang, Z.; Jiang, X. Y. A plasmonic nanosensor for immunoassay via enzyme-triggered click chemistry. *ACS Nano* **2014**, *8*, 12741-12747.

(11) Kokado, A.; Arakawa, H.; Maeda, M. New electrochemical assay of alkaline phosphatase using ascorbic acid 2-phosphate and its application to enzyme immunoassay. *Anal. Chim. Acta* **2000**, *407*, 119-125.

(12) Millan, J. L. Alkaline phosphatases: structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. *Purinergic Signal* **2006**, *2*, 335-341.

(13) Liu, Y.; Schanze, K. S. Conjugated polyelectrolyte-based real-time fluorescence assay for alkaline phosphatase with pyrophosphate as substrate. *Anal. Chem.* **2008**, *80*, 8605-8612.

(14) Sun, J.; Hu, T.; Xu, X. L.; Wang, L.; Yang, X. R. A fluorescent ELISA based on the enzyme-triggered synthesis of poly (thymine)-templated copper nanoparticles. *Nanoscale* **2016**, *8*, 16846-16850.

(15) Chen, X.; Chen, J.; Zhang, H. Y.; Wang, F. B.; Wang, F. F.; Ji, X. H.; He, Z. K. Colorimetric detection of alkaline phosphatase on microfluidic paper-based analysis devices. *Chin. J. Anal. Chem.* **2016**, *44*, 591-596.

(16) Bessey, O. A.; Lowry, O. H.; Brock, M. J. A method for the rapid determination of alkaline phosphates with five cubic millimeters of serum. *J. Biol. Chem.* **1946**, *164*, 321-329.

(17) Kind, P. R. N.; King, E. J. Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. *J. Clin. Pathol.* **1954**, *7*, 322-326.

(18) Breslow, R.; Katz, I. Relative reactivities of p-nitrophenyl phosphate and phosphorothioate toward alkaline phosphatase and in aqueous hydrolysis. *J. Am. Chem. Soc.* **1968**, *90*, 7376-7377.

(19) Berg, S.; Luneberg, E.; Frosch, M. Development of an amplification and hybridization assay for the specific and sensitive detection of mycoplasma fermentans DNA. *Mol. Cell. Probes* **1996**, *10*, 7-14.

(20) Walter, K.; Schütt, C. Alkaline phosphatase in serum: continuous assay. In *Methods of Enzymatic Analysis (Bergmeyer, H.U. ed) 2nd ed., Volume II*, 1974, pp 860-864.

(21) Gao, Z. Q.; Deng, K. C.; Wang, X. D.; Miro, M.; Tang, D. P. High-resolution colorimetric assay for rapid visual readout of phosphatase activity based on gold/silver core/shell nanorod. *ACS Appl. Mater. Interfaces* **2014**, *6*, 18243-18250.

(22) Sun, J.; Hu, T.; Chen, C.; Zhao, D.; Yang, F.; Yang, X. Fluorescence immunoassay system via enzyme-enabled in situ synthesis of fluorescent silicon nanoparticles. *Anal. Chem.* **2016**, *88*, 9789-9795.

(23) Tang, H. T.; Lunte, C. E.; Halsall, H. B.; Heineman, W. R. p-aminophenyl phosphate: an improved substrate for electrochemical enzyme immnoassay. *Anal. Chim. Acta* **1988**, *214*, 187-195.

(24) Seo, J.; Ha, H.; Park, S.; Haque, A. M. J.; Kim, S.; Joo, J. M.; Yang, H. Immunosensor employing stable, solid 1-amino-2-naphthyl phosphate and ammonia-borane toward ultrasensitive and simple point-of-care testing. *ACS Sen.* **2017**, *2*, 1240-1246.

(25) Glavan, A. C.; Christodouleas, D. C.; Mosadegh, B.; Yu, H. D.; Smith, B. S.; Lessing, J.; Fernandez-Abedul, M. T.; Whitesides, G. M. Folding analytical devices for electrochemical eLISA in hydrophobic R^H paper. *Anal. Chem.* **2014**, *86*, 11999-12007.

(26) Das, J.; Jo, K.; Lee, J. W.; Yang, H. Electrochemical immunosensor using p-aminophenol redox cycling by hydrazine combined with a low background current. *Anal. Chem.* **2007**, *79*, 2790-2796.

(27) Akanda, M. R.; Choe, Y. L.; Yang, H. "Outer-sphere to inner-sphere" redox cycling for ultrasensitive immunosensors. *Anal. Chem.* **2012**, *84*, 1049-1055.

(28) Labib, M.; Sargent, E. H.; Kelley, S. O. Electrochemical methods for the analysis of clinically relevant biomolecules. *Chem. Rev.* **2016**, *116*, 9001-9090.

(29) Xu, S.; Ouyang, W.; Xie, P.; Lin, Y.; Qiu, B.; Lin, Z.; Chen, G.; Guo, L. Highly uniform gold nanobipyramids for ultrasensitive colorimetric detection of influenza virus. *Anal. Chem.* **2017**, *89*, 1617-1623.

(30) Sun, J.; Wu, H. X.; Jin, Y. D. Synthesis of thiolated Ag/Au bimetallic nanoclusters exhibiting an anti-galvanic reduction mechanism and composition-dependent fluorescence. *Nanoscale* **2014**, *6*, 5449-5457.

(31) Sun, J.; Yang, F.; Zhao, D.; Chen, C. X.; Yang, X. R. Integrated logic gate for fluorescence turn-on detection of histidine and cysteine based on Ag/Au bimetallic nanoclusters-Cu^{2+} ensemble. *ACS Appl. Mater. Interfaces* **2015**, *7*, 6860-6866.

(32) Ryu, J. C.; Lee, K. J.; Lee, S. H. Synthesis of ketoconazole derivatives. *Bull. Korean Chem. Soc.* **2003**, *24*, 460-466. (and references therein).

(33) Josse, J. Constitutive inorganic pyrophosphatase of Escherichia coli. II. Nature and binding of active substrate and the role of magnesium. *J. Biol. Chem.* **1966**, *241*, 1948-1955.

(34) Zheng, F. Y.; Guo, S. H.; Zeng, F.; Li, J.; Wu, S. Z. Ratiometric fluorescent probe for alkaline phosphatase based on betaine-modified polyethylenimine via excimer/monomer conversion. *Anal. Chem.* **2014**, *86*, 9873-9879.

(35) Li, S. J.; Li, C. Y.; Li, Y. F.; Fei, J. J.; Wu, P.; Yang, B.; Ou-Yang, J.; Nie, S. X. Facile and sensitive near-infrared fluorescence probe for the detection of endogenous alkaline phosphatase activity in vivo. *Anal. Chem.* **2017**, *89*, 6854-6860.

(36) Deng, J. J.; Yu, P.; Wang, Y. X.; Mao, L. Q. Real-time ratiometric fluorescent assay for alkaline phosphatase activity with stimulus responsive infinite coordination polymer nanoparticles. *Anal. Chem.* **2015**, *87*, 3080-3086.

(37) Chinen, A. B.; Guan, C. M.; Ferrer, J. R.; Barnaby, S. N.; Merkel, T. J.; Mirkin, C. A. Nanoparticle probes for the detection of cancer biomarkers, cells, and tissues by fluorescence. *Chem. Rev.* **2015**, *115*, 10530-10574.

(38) Xuan, Z. H.; Li, M. M.; Rong, P. F.; Wang, W.; Li, Y. J.; Liu, D. B. Plasmonic ELISA based on the controlled growth of silver nanoparticles. *Nanoscale* **2016**, *8*, 17271-17277.

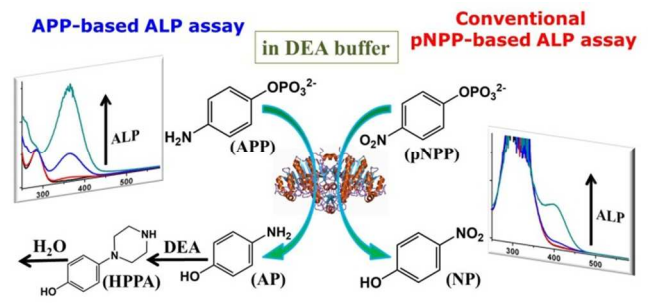
(39) Shang, L.; Dong, S. J. Design of fluorescent assays for cyanide and hydrogen peroxide based on the inner filter effect of metal nanoparticles. *Anal. Chem.* **2009**, *81*, 1465-1470.

(40) Liu, D. B.; Chen, W. W.; Wei, J. H.; Li, X. B.; Wang, Z.; Jiang, X. Y. A Highly sensitive, dual-readout assay based on gold nanoparticles for organophosphorus and carbamate pesticides. *Anal. Chem.* **2012**, *84*, 4185-4191.

(41) Liu, D. B.; Chen, W. W.; Tian, Y.; He, S.; Zheng, W. F.; Sun, J. S.; Wang, Z.; Jiang, X. Y. A highly sensitive gold-nanoparticle-based assay for acetylcholinesterase in cerebrospinal fluid of transgenic mice with Alzheimer's disease. *Adv. Healthc. Mater.* **2012**, *1*, 90-95.

(42) Shang, L.; Dong, S. J.; Nienhaus, G. U. Ultra-small fluorescent metal nanoclusters: synthesis and biological applications. *Nano Today* **2011**, *6*, 401-418.

(43) Sun, J.; Jin, Y. D. Fluorescent Au nanoclusters: recent progress and sensing applications. *J. Mater. Chem. C* **2014**, *2*, 8000-8011.


APP-based ALP assay
in DEA buffer
Conventional
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