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A Fluorescence Immunoassay Based on the Phosphate-Triggered Fluorescence Turn-on Detection of Alkaline Phosphatase

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ABSTRACT: A simple and cost-effective fluorescence immunoassay for the sensitive quantitation of disease biomarker α-fetoprotein (AFP) has been developed based on the phosphate-triggered fluorescence turn-on detection of alkaline phosphatase (ALP), with the reversible binding between calcein and Ce^{3+} as a signaling element. In this immunoassay, fluorescent calcein is readily quenched by Ce^{3+} via a coordination process. The ALP-catalyzed hydrolysis of *p*-nitrophenyl phosphate leads to the formation of *p*-nitrophenol and inorganic orthophosphate. And the newly formed orthophosphate could potently combine with Ce^{3+} due to the higher affinity, thus recovering the fluorescence of calcein. The corresponding fluorescence signal triggered by phosphate is related to ALP activities labeled on antibody, and thus could be applied to detect target antigen in an enzyme-linked immunosorbent assay (ELISA) platform. The fluorescence intensity correlated well to the AFP concentration ranges of 0.2−1.0 ng/mL and 1.0–4.0 ng/mL, with a detection limit of 0.041 ng/mL. The proposed fluorescence ELISA possesses convincing recognition mechanism and exhibits excellent assay performance in the evaluation of the AFP level in serologic test, which unambiguously reveals great application potential in the clinic diagnosis of disease biomarkers.

Enzyme-linked immunosorbent assay (ELISA) is mainly based on the highly specific antigen-antibody recognition and the efficient biocatalytic property of an enzyme.[1-2] By virtue of the excellent specificity, low cost and straightforward readout, ELISA has already become the most widely used format of immunoassay in clinical diagnosis,[2-4] foods quality control,[5-6] environmental monitoring,[7] and in laboratory research.[8] Alkaline phosphatase (ALP) is extensively used as a labeling enzyme in ELISA to produce detectable signals because of its high catalytic activity, broad substrate specificity, easy conjugation to antibodies, mild reaction conditions and good stability.[9] Moreover, ALP is a significant biomarker for diagnostics as well, and the abnormal level of ALP in serum is closely associated with several diseases, including prostatic cancer, bone disease, liver dysfunction and diabetes.[10] Therefore, highly sensitive and selective sensing of ALP activity is of great significance for both the ALP-related diseases diagnosis and the development of ALP-based ELISA platforms.

Until now, a large number of analytical methods for ALP activity determination have been developed, including colorimetry,[11-14] fluorometry,[15-20] chemiluminescence,[21] surface enhancement Raman scattering (SERS),[22] electrochemistry,[23-27] and chromatography.[28] Among these methods, fluorescence analysis methods have sparked significant excitement owing to their high sensitivity, cost effectiveness and convenience. The majority of the previous fluorescence approaches merely depend on the discrimination of pyrophosphate and phosphate by Cu^{2+}.[16, 29-30] As for ALP-based immunoassays, the routine procedures directly estimate the colored products of enzymatic hydrolysis by absorption spectroscopy or naked-eye, which usually suffer from poor sensitivity due to the limited amplification degree of detection signal. With the development of nanotechnology, several nanomaterial-related signal amplification strategies based on in-situ synthesis or aggregations of metal nanoparticles have been extended into the ALP-based plasmonic or colorimetric immunoassays.[8, 31-32] Though effective improvement in sensitivity has been achieved, the synthesis or surface modification of nanomaterials is ineluctably complicated, laborious and time-consuming. As a substitute of conventional colorimetric immunoassays, the fluorescent one is particularly appealing because of the intrinsic superior sensitivity and the foregoing advantages of fluorescence methods over traditionally used UV−vis spectrophotometry. However, currently reported ALP-based fluorescence immunoassays are quite limited and almost all rely on the ALP-enabled formation or fluorescence change of the fluorescent nanomaterials.[33-35] Therefore, despite many advances in the fields of ALP activity sensing and ELISA, it is still highly desirable to develop simple, rapid responsive, inexpensive and ultrasensitive ALP-based fluorescence ELISA protocols.

Fluorochrome calcein, as a cheap and commercially available fluorescent dye, possesses admirable yellow green fluorescence and excellent stability. The intense fluorescence of calcein could be quenched by several metal ions, and calcein has been devoted to fluorometric determination of certain metal ions and biomolecules.[36-39] In this regard, the specific reaction between calcein and Ce^{3+} would be particularly attractive and useful in the area of fluorescence sensing because the fluorescence of calcein can be dramatically quenched by Ce^{3+}.[40] On the other hand, phosphate, a universal hydrolysis product of phosphatase, could chelate with Ce^{3+}.[41] We can envision that the non-fluorescent calcein-Ce^{3+} complex would be applied to

assay phosphate-release reaction in a fluorescence enhancement manner based on the exchange of calcein by phosphate. As far as we know, there is scarcely any ALP sensing or/and ELISA system using calcein as an indicator up to now.

P-nitrophenyl phosphate (pNPP) is a frequently-used substrate of ALP, and the enzymatic hydrolysis of pNPP by ALP yields two products, p-nitrophenol (pNP) and phosphate ion. Conventionally, ALP activity is routinely estimated by measuring colorimetric or fluorescent signal difference between or induced by the substrate pNPP and product pNP.[15, 19] However, phosphate, as the other obvious enzymatic hydrolysate, is usually overlooked, which also has great potential to be employed to quantify ALP activity. Inspired by this, we have successfully designed a universal ALP-based fluorescence ELISA system based on phosphate-triggered fluorescence turn-on of the calcein-Ce^{3+} complex, with pNPP as the enzyme substrate. The extent of fluorescence enhancement directly hinges on the phosphate concentration generated from ALP reaction (i.e., the amount of ALP-secondary antibody conjugates), which is thereby indirectly related to the target antigen α-fetoprotein (AFP) concentration. Compared to the previous methods, this novel strategy shows several advantages. First, phosphate is the universal hydrolysis product of all the ALP substrates, therefore this protocol could be extended to other types of ALP-substrate systems and ALP-based ELISA. Second, besides the indispensable reagents for ELISA construction, no special and expensive reagents (only commercially available Ce^{3+} and calcein) are needed. Third, the fluorescent signal generation procedure could be performed in a facile mix-and-readout manner by simply mixing the enzyme reaction system with the Ce^{3+} and calcein, effectively avoiding complicated or time-consuming procedures. Finally, the assay is straightforward, selective and sensitive enough for practical application by means of the intrinsic high specificity of enzyme reaction, antigen-antibody recognition, and the intrinsic high sensitivity of fluorescent detection.

EXPERIMENTAL SECTION

Chemicals and Materials. Calcein was obtained from Aladdin Industrial Corporation (Shanghai, China). Alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa, 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP), adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), sodium pyrophosphate (Na_4PPi), bovine serum albumin (BSA), human serum albumin (HSA), lysozyme, peroxidase from horseradish (HRP), human immunoglobulin G (IgG), casein and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). Guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), 2'-deoxythymidine-5'-monophosphate (dTMP), were obtained from Sangon Biotechnology Co. Ltd. (Shanghai, China). α-fetoprotein (AFP), rabbit anti-AFP and mouse monoclonal antibody were bought from ProSpec (Ness Ziona, Israel). ALP-conjugated goat anti-rabbit secondary antibody was purchased from Abcam (Cambridge, MA). Both the wash buffer and antibody dilution buffer for ELISA were purchased from Boster (Wuhan, China). All reagents were analytical grade and used as received without any further purification. Ultrapure water from a Millipore system (resistivity 18.25 MΩ cm, Millipore, USA) was used throughout all experimental procedures.

Apparatus and Characterization. Fluorescence spectra were recorded on a Hitachi F-4600 spectrofluorometer (Tokyo, Japan). UV−vis absorption spectra were obtained with a Cary 500 UV−vis spectrophotometer (Varian).

Detection of ALP Activity. 100 μL of pNPP (100 μM), 100 μL of ALP with various activities, 50 μL of Ce^{3+} (100 μM) and 500 μL of ultrapure water were subsequently added into 200 μL of Tris-HCl (50 mM, pH 9.0) buffer solution, followed by incubation at 37°C for 30 min. Afterward, 50 μL of calcein (100 μM) was added into the above reaction solution, and the fluorescence intensities of resultant solutions were recorded directly.

Fluorescence Immunoassay for AFP. First, 100 μL of diluted mouse monoclonal antibody (1:200) in coating solution was added into the wells of a 96-well polystyrene plate and incubated at 4°C overnight. After discarding the solutions, the plate was washed three times with 300 μL of wash buffer (TBST), and blocked with 1% BSA at 37°C for 1 h to block nonspecific binding sites. After washing again, 100 μL of AFP standard solutions with various concentrations (0, 0.2, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 10, 20 ng/mL) were added into each well to incubate at 37°C for 1 h, followed by washing steps and the addition of rabbit anti-AFP (1:500, 100 μL). After incubation at 37°C for 1 h, the wells were washed three times, 100 μL of goat anti-rabbit secondary antibody labeled with ALP (1:3000) was added, and subsequently incubated at 37°C for 1 h. After washing the plate, we performed the enzymatic reaction as follows: 100 μL of pNPP (500 μM), 50 μL of Ce^{3+} (500 μM) and 50 μL of Tris-HCl (50 mM, pH 9.0) buffer solution were subsequently added into each well, and then incubated at 37°C for 30 min. Afterward, 50 μL of the above reaction solution was added into 950 μL of Tris-HCl (50 mM, pH 9.0) buffer solution containing calcein (62.5 μL, 100 μM), and the fluorescence intensities of resultant solutions were directly recorded.

Fluorescence Immunoassay for the Real Clinical Samples. Serum samples from two normal people and four patients who suffered from hepatocellular carcinoma (HCC) were kindly provided by the Second Hospital of Jilin University. The detailed procedures of the fluorescence ELISA referred to that for the model protein by just adding the diluted human serum instead of the AFP standard solution.

RESULT AND DISCUSSIONS

The Design and Establishment of the Sensing System for ALP Activity. Fluorescent calcein, could be efficiently quenched by combining with Ce^{3+} ion with 1:1 stoichiometry probably via an energy or charge transfer mechanism.[40, 42] The reaction could be reversed, thus offering the possibility of fluorescence turn-on assay of phosphate, which could form thermodynamically more stable complex with Ce^{3+} than calcein. Consequently, the calcein-Ce^{3+} system could be extended for ALP detection by means of the ALP-catalyzed generation of phosphate.

For an enzymatic reaction-based assay, high discrimination ability towards enzyme substrate and product is the necessary prerequisite to successfully detecting the corresponding enzyme activity. Therefore, we first evaluate the response signal of the as-proposed calcein-Ce^{3+} system toward various phosphate-containing molecules or ions, including phosphate, pNPP, AMP, ADP, ATP, GMP, GDP, GTP, UMP, UDP, UTP, dTMP and PPi (10 μM). The results in Figure 1A clearly imply that only phosphate could evoke significant fluorescence enhancement. This is ascribed to the more robust and stable complex of phosphate-Ce^{3+} than calcein-Ce^{3+} and the concomitant release of free fluorescent calcein from non-fluorescent calcein-Ce^{3+} complex. The response of other phosphate-

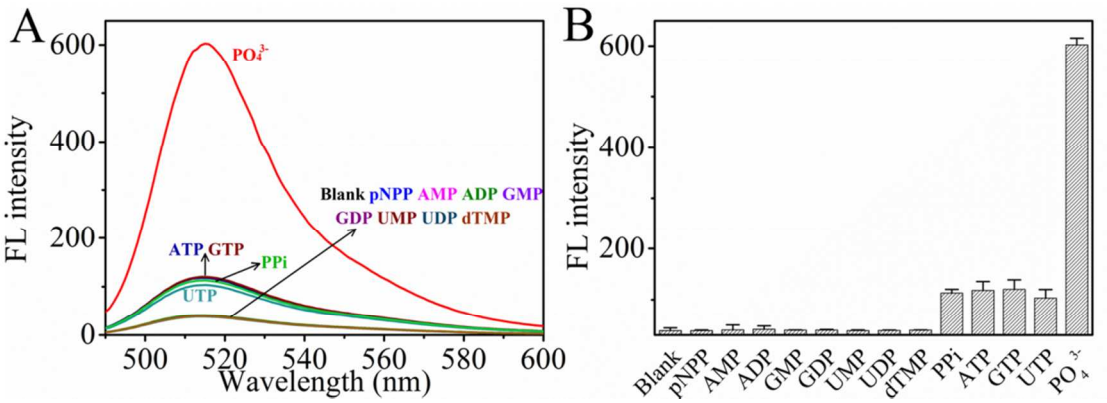

Figure 1. (A) Fluorescence spectra of the calcein-Ce^{3+} sensing system in the presence of various phosphate-containing targets (10 μM). (B) Relative responses of the phosphate-containing compounds compared to phosphate. Calcein, 5 μM; Ce^{3+}, 5 μM; pH 9.0 (10 mM Tris-HCl). λ_{ex} = 480 nm.

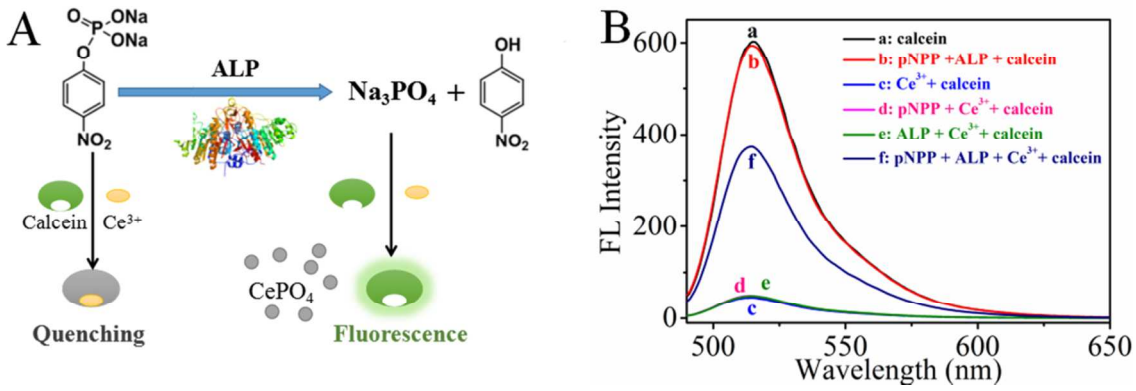

Figure 2. (A) Schematic illustration of ALP activity detection based on phosphate induced fluorescence turn-on of calcein-Ce^{3+} complex. (B) Fluorescence spectra of the calcein-Ce^{3+}-based sensing system in the presence of different reaction components. Calcein, 5 μM; Ce^{3+}, 5 μM; pNPP, 10 μM; ALP, 1 mU/mL; pH 9.0 (10 mM Tris-HCl); incubation at 37°C for 30 min. λ_{ex} = 480 nm.

containing compounds investigated could be ignored compared with that of PO_4^{3-}, although they possess similar phosphate group. Apparently, the aforementioned pNPP, AMP, ADP, ATP, GMP, GDP, GTP, UMP, UDP, UTP, dTMP and PPi molecules do not permit for valid interaction with Ce^{3+}.[43-45] As a result, the quenched fluorescence of calcein-Ce^{3+}complex could not be recovered by them. The high discrimination ability towards free phosphate and other phosphate-containing molecules or ions provides a sound basis for the fabrication of a versatile ALP assay, as well as the ALP-based fluorescence ELISA in the subsequent steps. In this study, pNPP is selected as a model ALP substrate for the sake of low background signal and ease of comparison with standard pNPP-based chromogenic method.

Figure 2A schematically illustrates the fluorescence strategy for ALP activity sensing based on the phosphate-induced fluorescence turn-on of calcein-Ce^{3+} complex. The organic phosphate group on the pNPP is unable to coordinate with Ce^{3+}, so the fluorescence of calcein-Ce^{3+} cannot be recovered in the presence of pNPP. However, the presence of ALP can catalyze the dephosphorylation of the pNPP to produce phosphate, which could combine with Ce^{3+} to form more stable $CePO_4$ (Ksp ~ 10^{-24}) complex compared to calcein-Ce^{3+} complex.[41] In this case, calcein is deprived of Ce^{3+} ion by newly generated phosphate, accompanied by the recovery of the fluorescence of Ce^{3+}-quenched calcein. The degree of fluorescence enhancement is related to ALP amount, which lays a foundation for assessing ALP activity.

As shown in Figure 2B, several tests are conducted to verify the feasibility of the proposed fluorescence turn-on assay for the sensitive quantitation of ALP activity. In this regard, calcein (5 μM) has an intense fluorescence emission peak centered at around 512 nm (line a, λ_{ex} = 480 nm), and the co-existence of ALP (1 mU/mL) and pNPP (10 μM) could not depress the fluorescence signal of calcein (line b), indicating the ALP-catalyzed dephosphorylation reaction system has a negligible effect on the fluorescence emission of calcein. As expected, when Ce^{3+} (5 μM) is introduced, the fluorescence signal of calcein is effectively quenched (line c). However, when Ce^{3+} is premixed with ALP-pNPP reaction solution, the fluorescence of calcein-Ce^{3+} is greatly enhanced (line f) due to the coordination of the newly formed phosphate with Ce^{3+}. In several other experiments, the pre-incubation of pNPP and Ce^{3+} could not influence the coordination of calcein with Ce^{3+} and the fluorescence quenching of calcein, which overwhelmingly confirms that the phosphate group on pNPP is incapable of coordinating with Ce^{3+} (line d). Moreover, the mixture of ALP with Ce^{3+} also invokes no fluorescence enhancement (line e). All these results undoubtedly reveal that, it is the ALP-catalyzed release of phosphate from the pNPP actuates the fluorescence enhancement.

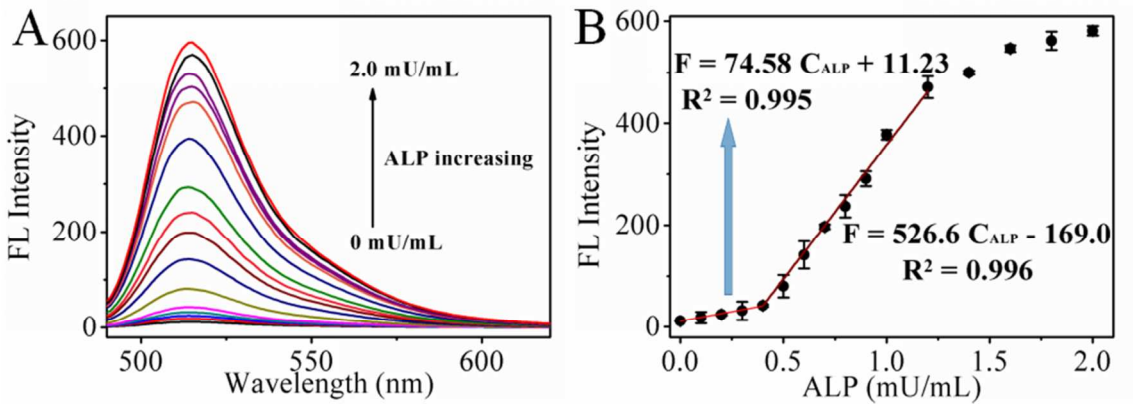

Figure 3. (A) The fluorescence emission spectra of the sensing system toward ALP with various activities (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 mU/mL). (B) The plots of fluorescence intensities at 512 nm versus the ALP activities. Calcein, 5 μM; Ce^{3+}, 5 μM; pNPP, 10 μM; pH 9.0 (10 mM Tris-HCl); incubation at 37°C for 30 min. λ_{ex} = 480 nm.

Fluorescence Turn-on Sensing of ALP Activity. Prior to assessing the capability of the proposed calcein-Ce^{3+} complex to detect ALP activity, we have optimized several experimental conditions like enzymatic reaction time, pH, pNPP concentrations and Ce^{3+} concentrations. Fascinatingly, premixing of Ce^{3+} with ALP-pNPP reaction solution would not impede the ALP-catalyzed dephosphorylation reaction (Figure S1). Thus it is possible to simultaneously conduct ALP-catalyzed dephosphorylation and $CePO_4$ formation reactions in one step, which makes the ALP analysis more simple and quick. And as a side benefit, since phosphate is a competitive inhibitor for the enzymatic reaction via occupying active site in ALP,[16, 46] the immediate consumption of generated phosphate by Ce^{3+} will accelerate the enzymatic reaction due to the restraint of phosphate inhibition. In this work, the fluorescence intensity of the analytical solution is increased gradually with increasing enzymatic reaction time, and levels off after 30 min, while the background hydrolysis reaction cannot proceed within as least 50 min (Figure S2). Thus, we choose 30 min as the optimum enzymatic reaction time. As is well-known, alkaline condition is propitious to the ALP-participated catalytic reaction, hence pH within the range from 7.5 to 9.5 is performed, and pH 9.0 is the optimal value in following experiments (Figure S3). The fluorescence intensity of calcein is directly modulated by Ce^{3+} level in this assay. To ensure a low background signal in the sensing system, 5 μM of Ce^{3+} (identical to the concentration of calcein) is used in further experiments. Because the detection range and sensitivity of an enzyme greatly depend on the substrate concentration, pNPP concentration is evaluated and optimized to be 10 μM (Figure S4).

Having ascertained these experimental conditions, the fluorescence response of the proposed sensing system toward ALP with various activities is studied and depicted in Figure 3. When ALP activity increases from 0 to 2.0 mU/mL, the fluorescence emission spectra centered at around 512 nm enhance gradually (Figure 3A) due to the increase of produced phosphate concentrations. Figure 3B shows the plots of fluorescence intensities at 512 nm versus the ALP activities. The fluorescence recovery correlates well to the ALP activities in the ranges of 0.1–0.4 mU/mL (R^2 = 0.995) and 0.4–1.2 mU/mL (R^2 = 0.996). The calculated detection limit is as low as 0.023 mU/mL based on 3σ/S, which is 1~2 order of magnitude better than the pNPP-based standard chromogenic method.[34] The sensitivity is also comparable with or superior to most previously reported methods (Table S1). In this work, we also have detected the ALP activity via estimating the colored product pNP to make a comparison with the fluorescence method. Both the absorption spectra and absorbance intensity at 405 nm increase progressively with the increase of ALP activities from 0.1 to 2.0 mU/mL (Figure S5). The detection limit is 0.13 mU/mL, which is 1 order of magnitude higher than that of the proposed fluorescence method. As the normal range of serum ALP in adults is reported to be about 40–190 mU/mL, the as-established fluorescence assay is sensitive enough for practical detection of ALP activity in biological samples. Besides high sensitivity, the intrinsic high specificity of enzyme reaction would confirm an ideal selectivity for ALP sensing. The developed fluorescence assay is simple, sensitive, selective and cost-effective to monitor ALP activity, which holds great promise for fluorescence immunoassays.

Investigations on the inhibitor of enzyme are of great importance in drug design. Therefore, besides the ability of ALP activity detection, this proposed fluorescence assay has been also utilized to evaluate the enzyme inhibition efficiency. As an acknowledged ALP inhibitor, Na_3VO_4 is employed for inhibiting assays. With the addition of Na_3VO_4 into the assay solution, the release of phosphate (i.e., hydrolysis of pNPP) is restricted due to inhibition of ALP activity (1.2 mU/mL), and the fluorescence recovery would be depressed accordingly (Figure S6A). Figure S6B manifests that the fluorescence intensity at 512 nm decreases progressively with the increase of Na_3VO_4 concentration from 0 to 1000 μM. The IC_{50} (the inhibitor concentration required for 50% inhibition of the enzyme activity) is calculated to be approximately 61.2 μM, which is in accord with the reported values determined by other works.[19, 47] These results clearly prove that our method is prospective for the screening of ALP inhibitors in drug discovery.

Fluorescence ELISA for Detection of AFP. Inspired by the successful and extensive use of ALP in ELISA, we proceed to investigate the potential application of the fluorescence turn-on ALP activity sensing system to ALP-labeled immunoassay for target antigen. AFP is taken as our model antigen considering its importance in hepatocellular carcinoma (HCC). In this case, the capture antibody and the primary antibody are mouse anti-AFP monoclonal antibody and rabbit anti-AFP, respectively, and the secondary antibody is goat anti-rabbit IgG labeled with ALP. The procedures of this fluorescence ELISA are nearly the same as the conventional ones.[48-49] Figure 4A schematically represents the ALP-labeled immunoassay for the detection of AFP. In this approach, the target AFP is first captured with specific antibodies pre-immobilized on a 96-well plate. Subsequently, rabbit anti-AFP and second antibody label

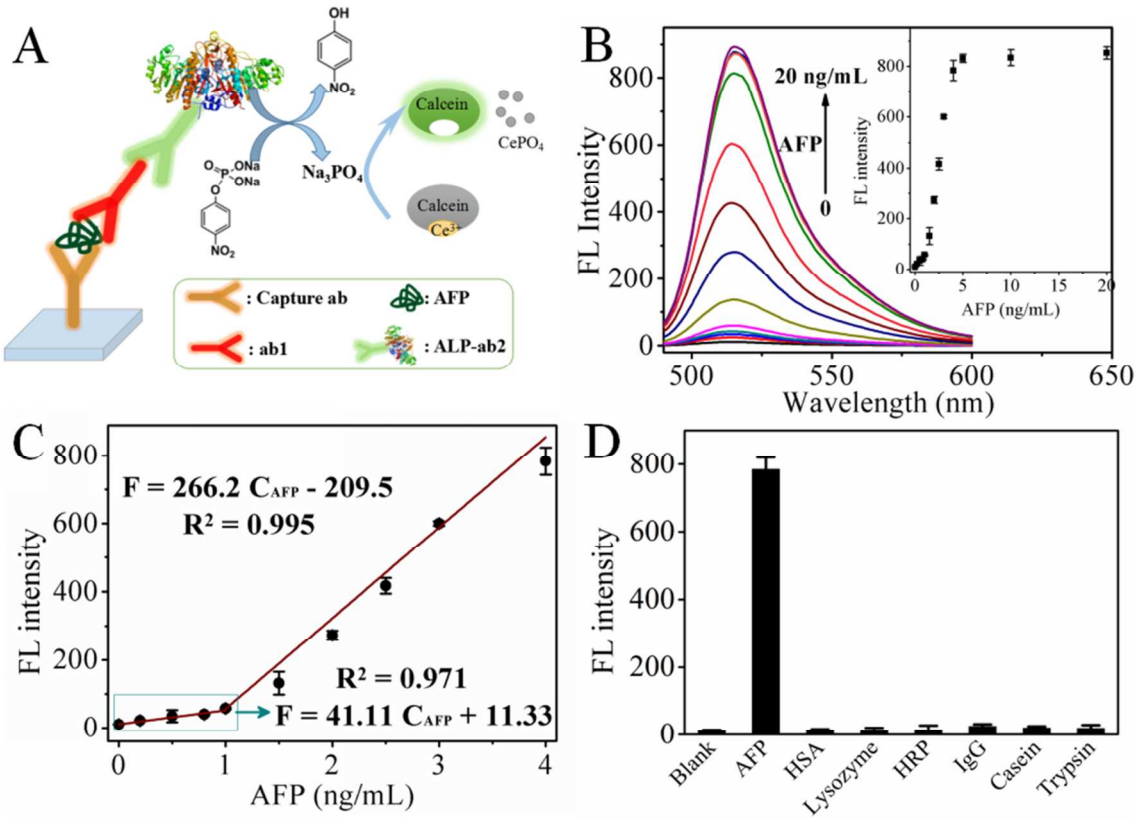

Figure 4. (A) Schematic representation of the fluorescence ELISA strategy via phosphate triggered fluorescence turn-on of the calcein-Ce^{3+} complex. (B) The fluorescence emission spectra and (insert) fluorescence intensities at 512 nm of the sensing system toward AFP with various concentrations. (C) The linear calibration plots for AFP detection. (D) Fluorescence responses of the developed ELISA against AFP (4 ng/mL) or other control enzymes/proteins (40 ng/mL). Experimental conditions for the enzymatic reaction: Calcein, 6.25 μM; Ce^{3+}, 6.25 μM; pNPP, 12.5 μM; pH 9.0 (10 mM Tris-HCl); incubation at 37°C for 30 min. λ_{ex} = 480 nm.

with ALP are successively immobilized on the plate via antigen-antibody specific recognition. After immobilization of these molecules on a 96-well plate following the procedures of conventional ELISA, pNPP and Ce^{3+} are added to generate orthophosphate and form $CePO_4$ simultaneously, then calcein is introduced, giving a fluorescent readout signal. To demonstrate the viability of our design, quantitative AFP detection experiment is implemented through our ALP-based ELISA system. The more AFP is introduced, the more ALP labeled on antibody would be banded. Therefore, the fluorescence intensities at 512 nm gradually increase when the AFP concentrations increase from 0 to 20 ng/mL (Figure 4B), exhibiting good linearity with AFP from 0.2 to 1.0 ng/mL (R^2 = 0.971) and from 1.0 to 4.0 ng/mL (R^2 = 0.995) (Figure 4C). The detection limit is 0.041 ng/mL calculated from 3σ/S, which is comparable with or better than other previously reported immunoassays for the detection of AFP (Table S2). The AFP concentration in normal subjects is less than 20 ng/mL, and abnormally elevated AFP (typically exceeds 400 ng/mL) is usually observed in the serum of patients with HCC. Obviously, this method could be available for the sensitive detection of disease biomarkers (AFP) in clinical applications by simply diluting real samples.

To verify the sensing ability of the assay in real samples, high specificity toward target antigen AFP is required. Therefore we challenge the ELISA platform with other nonspecifi- proteins, such as HSA, lysozyme, HRP, human IgG, casein, and trypsin. Because this proposed ELISA employs a particular antibody with specificity for AFP, it almost certainly has a splendid capability of selective recognizing AFP. Unsurprisingly, other examined proteins induce negligible change in fluorescence signal (Figure 4D), proving that our fluorescence immunoassay system is sufficiently selective for the detection of target AFP.

Encouraged by the high sensitivity and selectivity of this fluorescence ELISA, we apply it to the detection of target AFP in real samples. For this purpose, six different human serum samples from two normal adults and four patients with HCC are analyzed. The experiment is performed on the six unrelated human serum samples, and the results are shown in Table S3. As the AFP levels in serums of both normal adults (typically < 20 ng/mL) and patients with HCC (typically > 400 ng/mL) are outside of the linear range of our developed assay, the serums samples are diluted 10 and 200 times respectively to reduce AFP to an appropriate level. The results obtained from this assay are consistent well with the results from the pNPP-based standard ELISA method (Table S3), which verifies that our assay has a high accuracy and excellent performance in real sample analysis.

CONCLUSIONS

A versatile fluorescence biosensor for ALP that can be directly imposed on the traditional ELISA platform is established with improved performance. The proposed method is based on the phosphate-triggered fluorescence turn-on of the calcein-Ce^{3+} complex. It has proven to be straightforward, facile and cost-effective, and exhibits a much higher sensitivity compared with the traditional chromogenic assay for ALP. It also provides a potential platform for trace ALP inhibitor screening in drug discovery. Furthermore, using ALP as the labeling tracer and calcein as the signaling element, an ALP-based fluores-

cence turn-on ELISA platform for AFP detection is thereby developed. The detection limit is as low as 0.041 ng/mL, and the method shows impressive results in detecting target AFP in serum samples. Above all, since the assay is based on the fluorescence turn-on by dephosphorylation-released phosphate, and phosphate is the universal hydrolysis product of various ALP substrates in all ALP-participated enzymatic reaction, this protocol can be easily extended to other types of ALP-substrate systems. Therefore, other more cost-effective and easily achieved ALP substrates (e.g. PPi and ATP) than pNPP could be applied to such ALP-based ELISA system.

ASSOCIATED CONTENT

Supporting Information

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

Fluorescence spectra toward ALP with adding Ce^{3+} in different steps, optimization of enzymatic reaction time, pH values and pNPP concentration for ALP detection, detection of ALP using pNPP-based chromogenic method, comparison of various methods for the detection of ALP, fluorescence spectra and intensity of the sensing system in the presence of Na_3VO_4-treated ALP, comparison of various immunoassays for the detection of AFP and analytical results for AFP in the human serum samples (PDF).

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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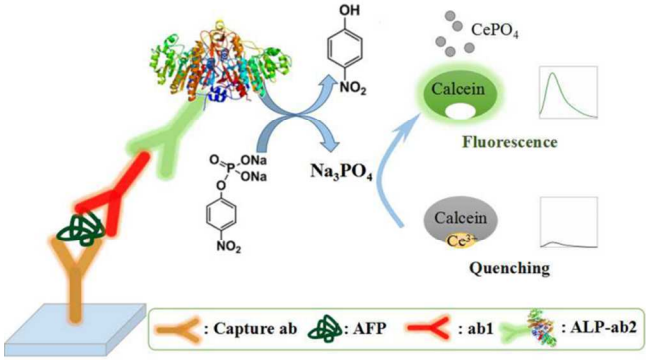
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